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TITLE: Regulation of Drug Sensitivity by Functional Status of p53 in Human Prostate Cancer

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13. ABSTRACT (Maximum 200 Words) Multidrug resistance protein MRP1 is overexpressed in advanced stage and grade human prostate cancer and is negatively regulated by p53. We found that antiandrogen flutamide is affected by MRP1 expression. There were significant differences between the sensitive and MRP1-overexpressing cells in efflux and accumulation of flutamide and hydroxyflutamide. Treating the cells with MRP1 modulators restored flutamide and hydroxyflutamide accumulation. Intracellular glutathione depletion with buthionine sulfoximine or energy depletion using 2-deoxy-D-glucose/sodium azide restored flutamide accumulation to that of parental cells. These studies indicate that flutamide and hydroxyflutamide are transported by MRP1 and that these findings may contribute to our understanding of resistance to hormone refractory prostate cancer. We also determined the effect of compounds that alter p53 function on MRP1 expression. We found that chlorpromazine, promazine, and <i>trans</i> -flupenthixol caused a 2-3-fold increase in wild-type p53 conformation and CP-31398 increased wild-type p53 conformation 6-10-fold. Promazine and chlorpromazine increased p21 in a dose-dependent manner. LVCaP cells exposed to CP-31398 showed a 5-fold increase in p21 and 10-fold decrease in MRP1. These data suggest that CP-31398 is an effective inducer of p21 expression and MRP1 repression in the prostate cancer cells and that compounds with similar structures and greater activity can be identified.		
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Table of Contents

Cover.....	1
SF 298.....	2
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	8
Reportable Outcomes.....	8
Conclusions.....	9
References.....	9
Appendices.....	10

INTRODUCTION

The treatment of recurrent prostate cancer is unsatisfactory. Despite an initial response to androgen-ablation therapies, even the most active cytotoxic drugs produce meaningful responses in <50% of patients. We now know each cancer has a set of genetic determinants that may influence the biology of the disease and the response to treatment. The *p53* tumor-suppressor gene is commonly mutated in human prostate cancer and may play an unexpectedly important role in drug resistance (1). *p53* is implicated in the response to cellular damage through its central role in growth arrest and apoptosis. Cells with wild-type *p53* have a propensity towards cell-cycle arrest following DNA damage, whereas cells with mutant *p53* appear to bypass the G1/S check point. The former response is believed to allow time for repair of cellular damage, whereas the latter permits DNA replication at a time when the viability of cells is questioned. Under circumstances of either overwhelming cellular damage or conflicting cellular signals, wild-type *p53* may direct cells down an apoptotic pathway (2). In contrast, cells with mutant *p53* appear less likely to undergo apoptosis at equivalent levels of injury. To understand the basis for this multidrug resistance phenotype, we studied the sequential expression of several determinants of drug sensitivity in 95 human prostate cancers. We found a non-random increase in several important proteins including *p53*, topoisomerase IIa, and MRP1. We were particularly struck by the almost parallel increase in *p53* staining and MRP1 staining in these cases. We find that there appears to be a co-regulation of *p53* and MRP1, the multidrug resistance protein, during the progression of prostate cancer from low stage disease to high stage disease. In addition, we demonstrated that the transcriptional status of *p53* could determine the level of expression of MRP1. Therefore, we proposed to investigate the importance of *p53* in prostate cancer multidrug resistance and determine if restoration of *p53* function by small molecule chemicals can restore drug sensitivity. Specifically, we studied the role of MRP1 in resistance to hormonal and chemotherapy and whether pharmacological stabilization of wild-type *p53* and rescue of mutant *p53* can suppress the expression of MRP1 and sensitize cells to chemotherapy.

BODY

Task 1. To determine the role of MRP1 and MAP-4 in resistance to hormonal and chemotherapy.

Prostate cancer is the most common noncutaneous malignancy of American men. Although it can be initially treated with androgen deprivation therapy, tumors that relapse become resistant to future hormonal manipulation. Flutamide is a nonsteroidal antiandrogen that acts as a competitive inhibitor of dihydrotestosterone for the androgen receptor and is often used as a part of initial treatment. Flutamide and its active metabolite hydroxyflutamide block expression of genes with promoters that contain androgen response elements and also prevent androgen-dependent stabilization of the androgen receptor. Although >50% of patients initially respond to androgen deprivation, most patients relapse within a median of 12–18 months, at which time, they are resistant to additional hormonal therapy. Retreatment with antiandrogen therapy or chemotherapy of patients with hormone-refractory disease has not been shown to significantly improve the overall survival of 2–3 years.

We previously found that the multidrug resistance protein (MRP), MRP1, is overexpressed in advanced stage and grade human prostate cancer and is negatively regulated by *p53*. MRP1 is a *M*r

190,000 membrane protein belonging to the ABC family of transporters. MRP1 pumps negatively charged substrates from the cytosol to the extracellular environment. Cotransport with glutathione (e.g., doxorubicin and vincristine) or conjugation with glutathione, glucuronides, or sulfates (e.g., leukotrienes) can enhance transport of MRP1 substrates. The structural features of drugs that are transported by ABC transporters have been extensively studied. We noted that the antiandrogen flutamide shares several of these characteristics, including a weak negative charge and an electron-withdrawing trifluoro group attached to a hydrophobic ring. Therefore, we determined whether the cellular accumulation of the antiandrogen flutamide, a drug commonly used in the treatment of prostate cancer, is affected by MRP1 expression.

To determine the effect of MRP1 overexpression on flutamide accumulation, we used cell lines that overexpress MRP1 either by transfection or selection. Figure 1 shows the overexpression of MRP1 as compared to the parental controls, and the lack of detectable P-glycoprotein in the drug-resistant cell lines. To assess whether overexpression of MRP1 changes flutamide accumulation, we compared the accumulation of [³H]-flutamide, [³H]-hydroxyflutamide, and [³H]-dihydrotestosterone in MRP1-overexpressing cells to that of parental cell lines. Figure 2 demonstrates that KB4D-10 and PC-3-ADR cells accumulated significantly less [³H]-flutamide (Figure 2a) and [³H]-hydroxyflutamide (Figure 2b) than the parental controls. This difference was observed after 15 seconds of incubation; steady-state accumulation was achieved after one minute (Figure 2 and Table 1). In contrast, the accumulation of [³H]-dihydrotestosterone was not affected by MRP1 expression (Figure 2c and Table 1).

To verify that the decrease in flutamide accumulation in MRP1 cells was energy dependent, we pre-incubated cells with sodium azide and 2-deoxy-D-glucose for 30 minutes. Under these conditions, steady-state accumulation of flutamide and hydroxyflutamide were restored to that of the parental cells (Figure 3a, b). In addition, incubation of the cells at 4°C abolished the MRP1-mediated flutamide transport (Figure 4a, b). In both instances, these conditions had no effect on accumulation of flutamide and hydroxyflutamide in sensitive cells (data not shown).

We next asked whether the difference observed in steady-state drug accumulation in MRP cells was due to changes in drug efflux. Figure 5 shows that the efflux of [³H]-flutamide (Figure 5a) and [³H]-hydroxyflutamide (Figure 5b) was significantly increased in cell lines overexpressing MRP1.

Leukotriene D4 enters cells passively, is transported by MRP1, and can competitively block the transport of MRP1 substrates (3). To test whether leukotriene D4 could compete for [³H]-flutamide and [³H]-hydroxyflutamide transport, cell lines were incubated with the anti-androgens after a three-hour pre-incubation with leukotriene D4. Figure 6 (a, b) demonstrates that leukotriene D4 restored the accumulation of flutamide and hydroxyflutamide in cell lines overexpressing MRP1 but had no effect on the accumulation of the anti-androgens in parental cells.

Glutathione enhances transport of MRP substrates by either direct conjugation or co-transport. To test the effects of glutathione on flutamide transport, we depleted cellular glutathione with BSO and measured the effect on flutamide accumulation. As shown in Figure 7 (a, b), BSO increased the accumulation of flutamide in MRP1 overexpressing cell lines but had no effect on parental cells.

To determine if VX-710, an inhibitor of MRP1, affected the transport of the clinically active agent hydroxyflutamide, we studied its effect on hydroxyflutamide accumulation. Figure 8 demonstrates that VX-710 increased the accumulation of hydroxyflutamide in a dose-dependent manner. There was no effect of the drug on the transport of hydroxyflutamide in wild-type cells. These results were published in *Cancer Research* (4).

Task 2. To determine whether pharmacological stabilization of wild-type p53 and rescue of mutant p53 can suppress the expression of MRP1 and MAP-4 and sensitize cells to chemotherapy.

Overexpression of MRP1 in prostate cancer results in decreased accumulation of and sensitivity to chemotherapeutic drugs. Currently, the approach to circumvent MRP1-mediated drug resistance is to use inert modulators that bind to the MRP1 protein, block its ability to transport chemotherapeutics and increase its intracellular drug concentrations. Although new drugs have been developed with greater specificity, this strategy has met with limited success with most regimens having little effect on drug sensitivity and clinical prognosis.

The *MRP1* gene was shown to interact with multiple transcription factors, such as Sp1, CRE, AP-1, AP-2 and MYCN. In addition, the expression of MRP1 can be repressed by proteins that do not bind to the MRP1 promoter. For example, we showed that p53 transcriptionally represses MRP1 and this can lead to an increase in intracellular drug concentrations (5). The tumor suppressor p53 acts as a transcription factor that is mutated in 50% of all human cancers. In addition, we have demonstrated that the frequency of p53 mutations increase from low to high-grade prostate cancer (6). Therefore, circumventing p53 mutations in prostate cancer may result in repressing MRP1 and increasing sensitivity to chemotherapeutic drugs.

The most frequent mutations in p53 involve point mutations in the p53 DNA binding domain. These mutations result in a change in conformation in the DNA binding domain and the inability for p53 to bind to its corresponding p53 response elements. Therefore, manipulating these mutations could be an effective approach for restoring p53 function. Previous studies have attempted to manipulate the DNA binding domain in order to upregulate p53-regulated genes. One approach is to use peptide fragments such as FL-CDB3 that can bind to the destabilized p53. Another technique is to use small molecules that can bind to p53 (7) that can have the advantages of being used in the clinic. The compound CP-31398 was identified from series of small molecules to rescue and stabilize wild-type p53 from the mutant protein by changing the DNA binding domain of the protein (7). The stabilization of the DNA binding domain results in the prevention of p53 degradation and changes the expression of p53-regulated genes involved in cell cycle arrest and apoptosis. The characteristics of these compounds are that they contain a hydrophobic core with a positively charged group connected by a 3-carbon linker (7). Our lab previously discovered that phenothiazines can modulate MDR1-mediated drug resistance, and these compounds share the structure similarities to CP-31398 (8). However, whether or not CP-31398 or these structurally related compounds can represses MRP1 has not been determined. Therefore, we studied whether CP-31398 and structurally similar compounds could repress MRP1 by p53 stabilization and restore intracellular drug concentrations.

Phenothiazines share some structural similarities to CP-31398 (8). To test if these compounds can stabilize the wild-type p53 conformation, an assay was developed to detect the wild-type p53

conformation by using antibodies specific for the wild-type p53 conformation and performing an ELISA (Figure 9). To verify that this assay can measure the wild-type p53 conformation, we heated the lysates from LVCaP cells at 38°C and performed the ELISA. Figure 10 demonstrates that heating the LVCaP lysate decreased the wild-type p53 conformation. In addition, we incubated the p53 conformation negative mutant R175H with CP-31398 and performed the ELISA. Figure 11a demonstrates that this compound can increase the wild-type p53 conformation in R175H cells. To determine if compounds with structural similarities to CP-31398 can stabilize the p53 wild-type conformation, we incubated the cells with phenothiazines and performed an ELISA. Figure 11b shows that promazine and chlorpromazine caused a 2-3-fold increase in the wild-type conformation. We then incubated LNCaP cells with the most potent compound chlorpromazine and examined p53 and p21 expression. Figure 12 demonstrates that p21 and p53 expression remain unchanged in LNCaP while p21 slightly increases in LVCaP.

To determine if CP-31398 can repress MRP1 at protein level, cells that harbor mutant (LVCaP) and wild-type (LNCaP) p53 were incubated with CP-31398. MRP1 was decreased by CP-31398 in a dose-dependent manner in LNCaP and LVCaP cells with maximum repression at 40 μ M (Figure 13). To determine the time course of MRP1 repression with CP-31398, LNCaP and LVCaP cells were exposed to 40 μ M CP-31398 over a period of 12 hours. Forty micromolar of CP-31398 represses MRP1 at 6 hours in LNCaP cells while CP-31398 represses MRP1 in less than 3 hours in LVCaP cells (Figure 14).

CP-31398 has been shown to stabilize p53 in mutant and wild-type cells (7). The expression of p53 expression was measured after CP-31398 exposure. Figures 13 and 14 show that CP-31398 upregulated p53 in LNCaP cells in a dose and time dependent manner but not in LVCaP cells. To determine if p53 activity was intact, the expression of the p53 regulated gene p21 was examined. Figure 13 and Figure 14 demonstrate that CP-31398 increases p21 expression in both LNCaP and LVCaP cells in a dose and time dependent manner.

The tumor suppressor p53 represses MRP1 transcription (5). To determine if MRP1 repression was due to transcriptional repression, cells were exposed to CP-31398 and mRNA expression was determined by semi-quantitative RT-PCR. Figure 15 demonstrates that MRP1 mRNA decreased slightly in LNCaP and LVCaP cells.

MRP1 mediates multidrug resistance by actively effluxing compounds from cells. To test if MRP1 repression by CP-31398 influences intracellular drug concentration, LNCaP and LVCaP cells were treated with CP-31398 and etoposide accumulation was determined. Figure 16a demonstrates that CP-31398 can increase intracellular concentrations of etoposide in LNCaP and LVCaP cells as compared to their untreated controls. However, when the cells were incubated with the MRP1 substrate Leukotriene C4, there was a significant increase in leukotriene accumulation in LNCaP but not in LVCaP cells (Figure 16b). To determine if CP-31398 was a substrate of MRP1, drug sensitivity was examined using MRP1 overexpressing cells and their parental controls. Figure 17 demonstrated that CP-31398 was not a substrate for MRP1.

To determine if the difference in drug accumulation and protein expression between the two cell lines was due to a change in the cell viability, LNCaP and LVCaP cells were treated with CP-31398

and viability was measured by trypan blue. Figure 18 demonstrated that CP-31398 decreased cell viability in LVCaP cells but not in LNCaP cells.

To determine if chlorpromazine represses MRP1 we incubated the cells with chlorpromazine and detected MRP1 expression with Western blot. Figure 19a demonstrated that chlorpromazine repressed MRP1 in a dose-dependent manner. To determine the time course of MRP1 repression, we incubated the cells with 40 μ M of chlorpromazine over a period of 12 hours. Figure 19b demonstrated that incubation with chlorpromazine repressed MRP1 at the same rate in both LNCaP and LVCaP cells. To determine if there is an effect on cell viability, the cells were treated with chlorpromazine and cell viability was measured by trypan blue exclusion. Figure 20 demonstrated that chlorpromazine decreased cells viability about in both LNCaP and LVCaP cells. To determine if chlorpromazine was a substrate of MRP1, drug sensitivity was examined using MRP1 overexpressing cells and their parental controls. Figure 17 demonstrated that chlorpromazine was not a substrate for MRP1. These results indicate that CP-31398 and the compounds with similar structure may be used to recover p53 function and restore chemotherapy sensitivity to cancer cells, and provides a clue for further drug development

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that MRP1 is overexpressed in human prostate cancer cells.
- Elucidated the role of MRP1 in resistance to antiandrogen drug flutamide.
-
- Discovered that MRP1 is negatively regulated by p53 in prostate cancer cells.
- Corroborate the contribution of p53 status to drug resistance in prostate cancer.
- Established an ELISA assay that can detect wild-type and mutant p53 conformation.
- Showed that pharmacological stabilization of wild-type and rescue of mutant p53 by small molecule compounds such as CP-31398 can suppress MRP1 expression and sensitize cancer cells to chemotherapeutic drugs such as etoposide (VP-16).

REPROTABLE OUTCOMES

Manuscripts

Grzywacz MJ, Yang JM, Hait WN: Effect of Overexpression of the Multidrug Resistance Protein (MRP1) on the Transport of the Anti-androgen Flutamide. *Cancer Res* 63(10): 2492-2498, 2003.

Grzywacz MJ, Yang JM, Hait WN: Repression of multidrug resistance protein 1 (MRP1) by pharmacologic restoration of p53 function in human prostate cancer cells. In preparation.

Abstracts

Grzywacz MJ, Yang JM, Hait WN: Overexpression of the Multidrug Resistance Protein (MRP1) Produces Resistance to Estramustine and Flutamide. *Proc Amer Assoc Cancer Res* 42: 1511, 2001.

Grzywacz MJ, Yang JM, Hait WN: The Active Metabolite of Flutamide, 2-hydroxyflutamide, is a substrate for MRP1. *Proc Amer Assoc Cancer Res* 44: 3685, 2003.

Grzywacz MJ, Yang JM, Hait WN: Repression of multidrug resistance protein 1 (MRP1) by pharmacologic alteration in p53 function in human prostate cancer cells. *Proc Amer Assoc Cancer Res* 45: 2136, 2004.

Degree obtained that are supported by this award

Matthew Grzywacz has been supported by this grant and will receive a Ph.D. degree from this work.

CONCLUSIONS

We have shown that antiandrogen flutamide, a drug commonly used in the treatment of prostate cancer, is transported by MRP1, based on drug accumulation and efflux studies and the ability of leukotriene D4, BSO, and VX-710 to restore steady-state concentrations. In contrast, transport of dihydrotestosterone was not affected by MRP1. These results suggest an unexpected role of MRP1 in the process of resistance to antiandrogen therapy of prostate cancer that could potentially be addressed through the use of MRP1 modulators. These findings may also contribute to our understanding of resistance to hormone refractory prostate cancer.

We have demonstrated that the p53-stabilizing compound CP-31398 and structurally related compounds could suppress MRP1 expression. The inhibition of MRP1 expression led to increases in intracellular drug accumulation and sensitivity to chemotherapeutic agents. These studies fortify the basis for a new strategy to overcome drug resistance caused by MRP1 expression and p53 mutation, and provide some clues for drug development.

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APPENDICES

Appendix I Figures and Tables

Appendix II Publications

APPENDIX 1

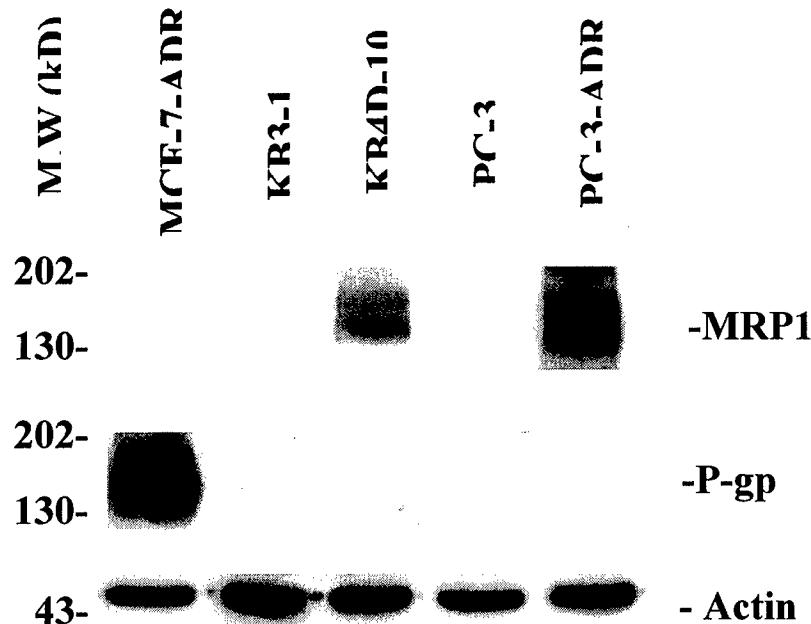


Figure 1. Expression of MRP1 in KB4D-10 and PC-3-ADR cancer cell lines. Fifty μ g of cell lysate from each cell line were resolved on an 8% SDS-PAGE and probed with antibodies to MRP1 (QCRL-1; Signet Dedham, MA), β -actin (Sigma, St Louis, MO) or P-glycoprotein (Signet, Dedham, MA). The results are a representative of three separate experiments.

Table 1. Steady-state accumulation of flutamide, hydroxyflutamide, and dihydrotestosterone in sensitive and MRP1 cell lines

Cell line	Flutamide	Hydroxyflutamide	Dihydrotestosterone
(pmol/10 ⁶ cells)			
PC-3	7.4 ± 0.3 *	4.6 ± 0.6 *	1.1 ± 0.1
PC-3-ADR	5.9 ± 0.8	3.1 ± 0.6	1.2 ± 0.2
KB3-1	6.9 ± 0.9 **	5.2 ± 0.6 *	1.4 ± 0.6
KB4D-10	4.5 ± 0.7	3.9 ± 0.5	1.7 ± 0.3

5.0×10^4 - 1.0×10^5 cells/mL/well were seeded onto 24 well plates and allowed to grow for 48 hours. The cells were washed with serum-free media then [³H]-flutamide, [³H]-hydroxyflutamide, or [³H]-dihydrotestosterone were added and incubated for 30 minutes. Drug accumulation was measured as described in "Materials and Methods". Each value represents the mean ± standard deviation of quadruplicate determinations (*-p<0.05, **-p<0.01 comparing the accumulation of drugs in MRP1 overexpressing cell lines to that of the parental controls).

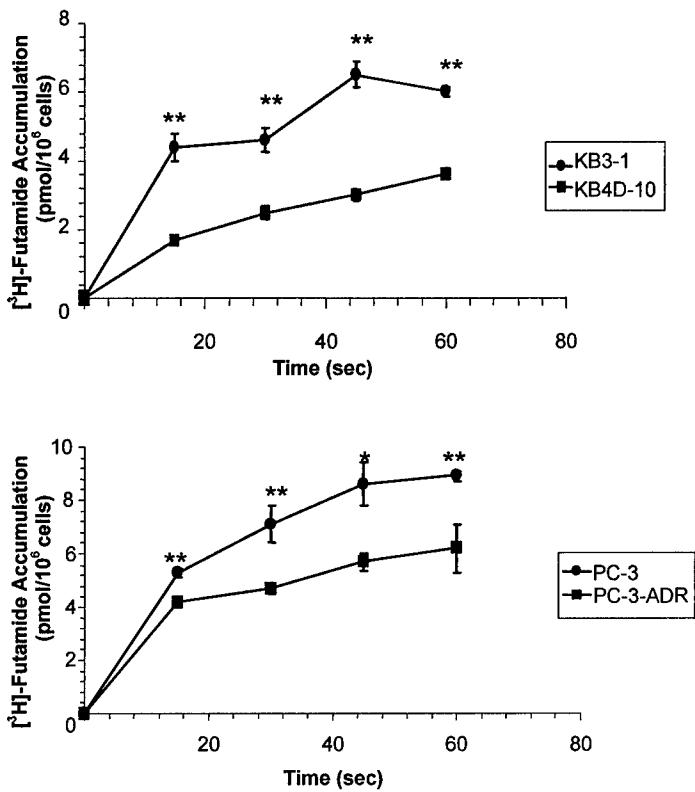


Figure 2a. Effect of MRP1 expression on flutamide accumulation. KB3-1, KB4D-10 (top) and PC-3, PC-3-ADR (bottom) were seeded onto 24 well plates, and 60 nM of $[^3\text{H}]\text{-flutamide}$. The cells were incubated 15 seconds to one minute then washed in ice-cold PBS, lysed with SDS, and measured for radioactivity by scintillation counting. Each point represents the mean \pm standard deviation of quadruplicate determinations. The results are a representative of three separate experiments (*- $p < 0.05$, **- $p < 0.01$, MRP1 expressing cells vs. parental cells).

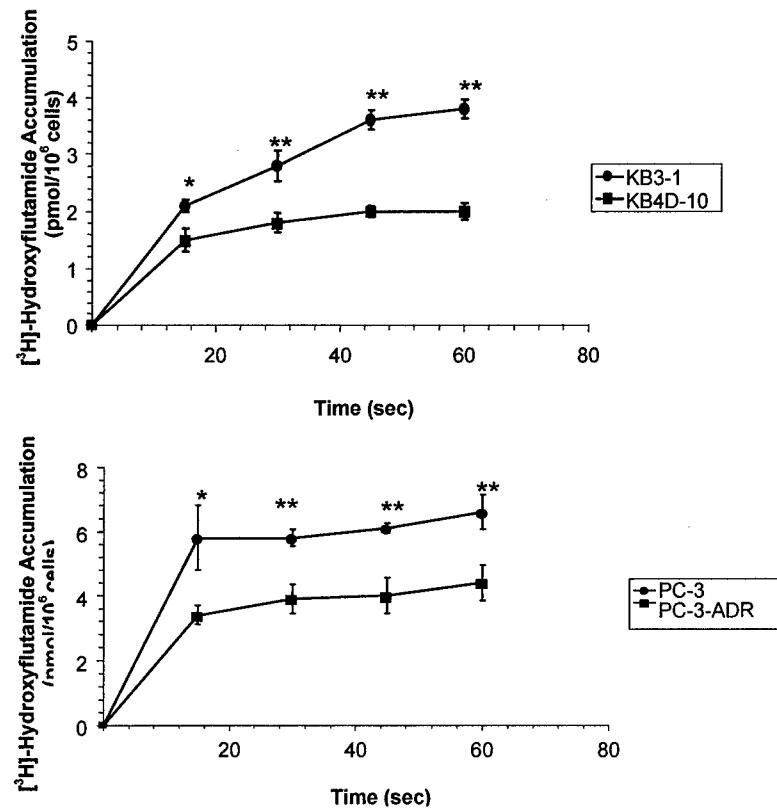


Figure 2b. Effect of MRP1 expression on hydroxyflutamide accumulation. KB3-1, KB4D-10 (top) and PC-3, PC-3-ADR (bottom) were seeded onto 24 well plates and 60 nM of $[^3\text{H}]\text{-hydroxyflutamide}$, was added to each well 48 hours later. The cells were incubated 15 seconds to one minute then washed in ice-cold PBS, lysed with SDS, and measured for radioactivity by scintillation counting. Each point represents the mean \pm standard deviation of quadruplicate determinations. The results are a representative of three separate experiments (*-p<0.05, **-p<0.01, MRP1 expressing cells vs. parental cells).

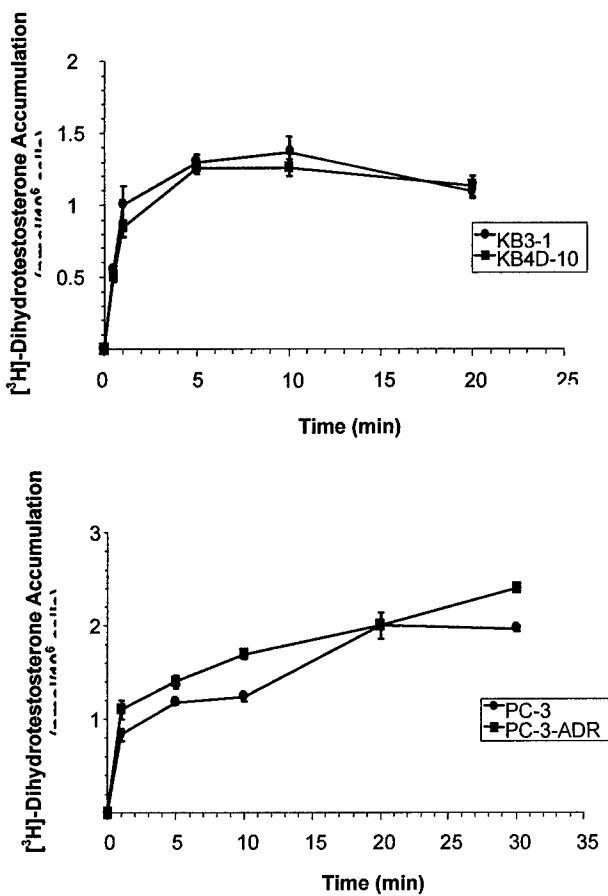


Figure 2c. Effect of MRP1 expression on dihydrotestosterone accumulation.

KB3-1, KB4D-10 (top) and PC-3, PC-3-ADR (bottom) were seeded onto 24 well plates, and 20nM of [³H]-dihydrotestosterone was added to each well 48 hours later. The cells were incubated 15 seconds to one minute then washed in ice-cold PBS, lysed with SDS, and measured for radioactivity by scintillation counting. Each point represents the mean \pm standard deviation of quadruplicate determinations. The results are a representative of three separate experiments (*-p<0.05, **-p<0.01, MRP1 expressing cells vs. parental cells).

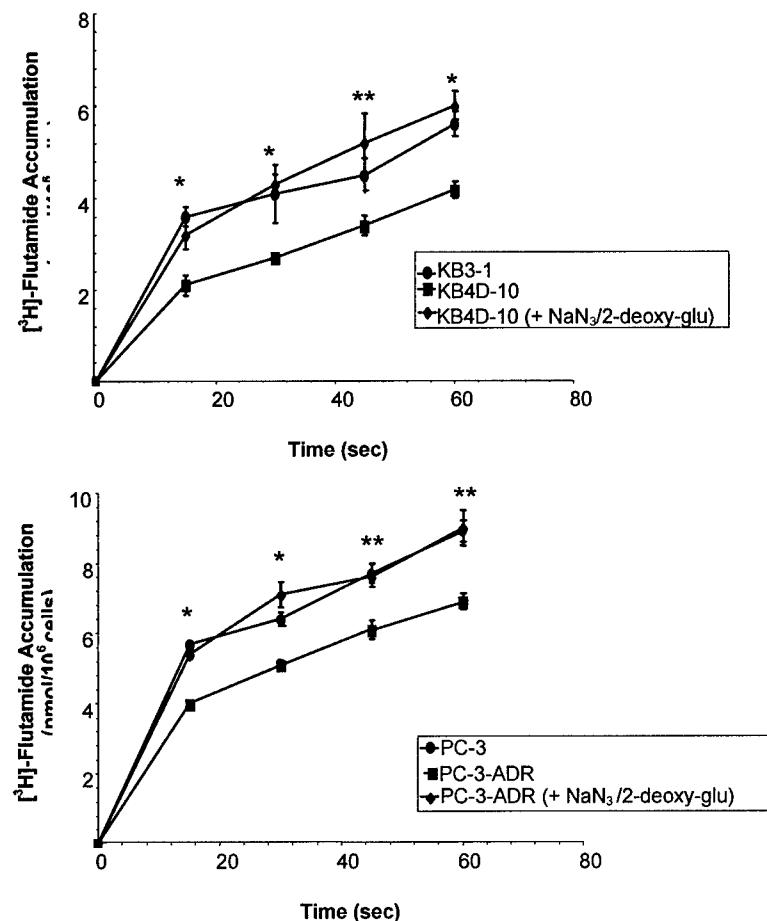


Figure 3a. Effect of sodium azide and 2-deoxy-D-glucose on flutamide accumulation. KB3-1, KB4D-10 (top) and PC-3, PC-3-ADR (bottom) were seeded onto 24 well plates. Forty-eight hours later the cells were incubated for 30 minutes in glucose-free media supplemented with 15mM sodium azide and 50 mM 2-deoxy-D-glucose. The accumulation of $[^3\text{H}]\text{-flutamide}$ was assayed as described in "Materials and Methods". Each point represents the mean \pm standard deviation of quadruplicate determinations. The results are a representative of three separate experiments (*-p<0.05, **-p<0.01, MRPI cells treated vs. untreated).

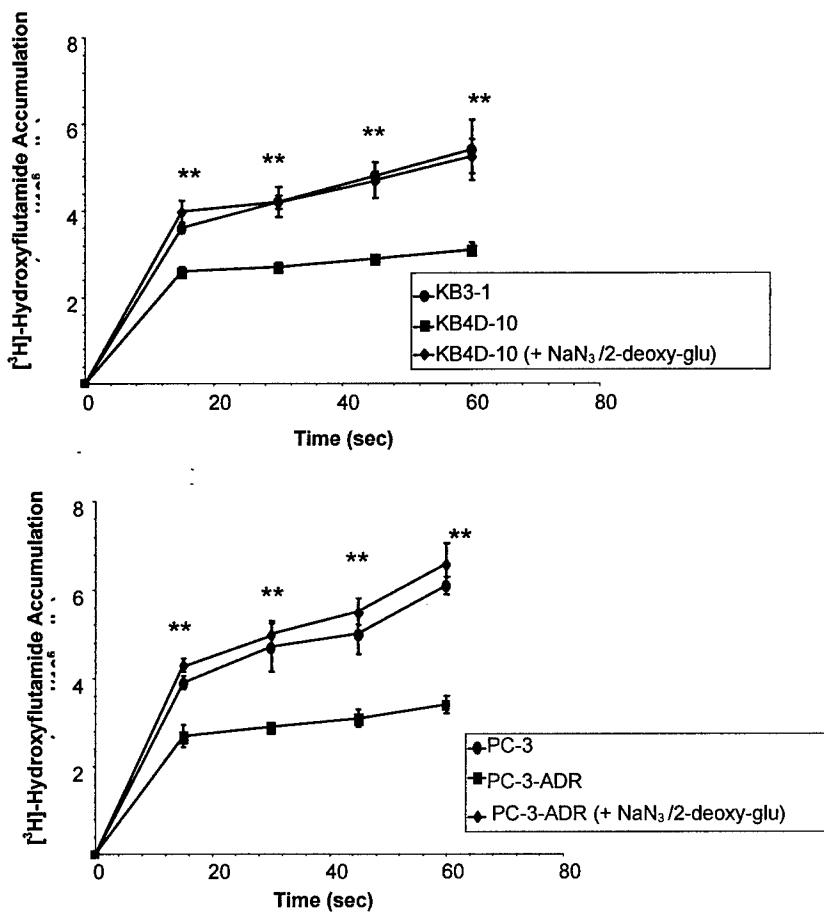


Figure 3b. Effect of sodium azide and 2-deoxy-D-glucose on hydroxyflutamide accumulation. KB3-1, KB4D-10 (top) and PC-3, PC-3-ADR (bottom) were seeded onto 24 well plates. Forty-eight hours later the cells were incubated for 30 minutes in glucose-free media supplemented with 15mM sodium azide and 50 mM 2-deoxy-D-glucose. The accumulation of [³H]-hydroxyflutamide was assayed as described in "Materials and Methods". Each point represents the mean \pm standard deviation of quadruplicate determinations. The results are a representative of three separate experiments (*-p<0.05, **-p<0.01, MRP1 cells treated vs. untreated).

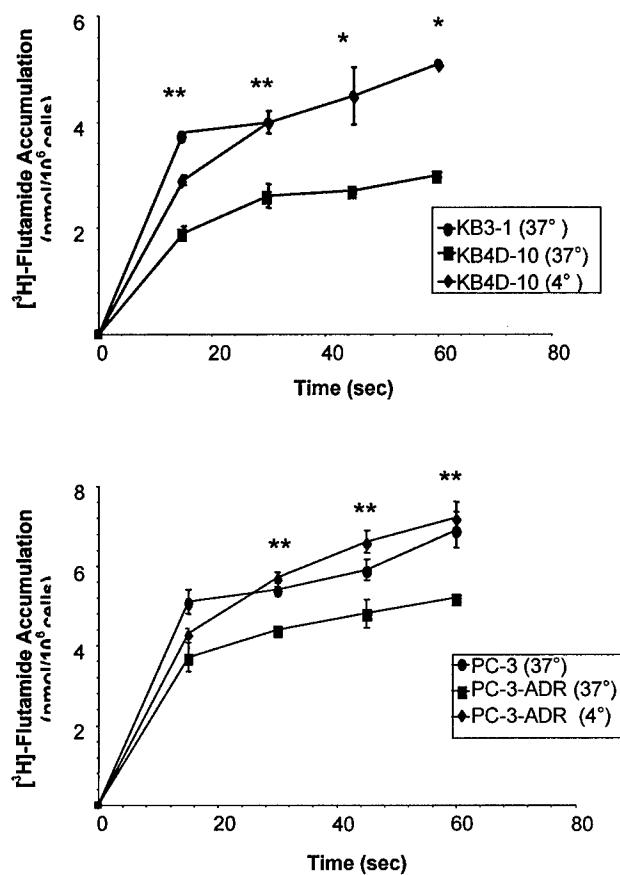


Figure 4a. Effect of temperature on flutamide accumulation. KB3-1, KB4D-10 (top) and PC-3, PC-3-ADR (bottom) were seeded onto 24 well plates. Forty-eight hours later the cells were incubated for one hour at 4°C. The accumulation of [³H]-flutamide was assayed as described in "Materials and Methods". Each point represents the mean \pm standard deviation of quadruplicate determinations. The results are a representative of three separate experiments (*-p<0.05, **-p<0.01, MRP1 cells treated vs. untreated).

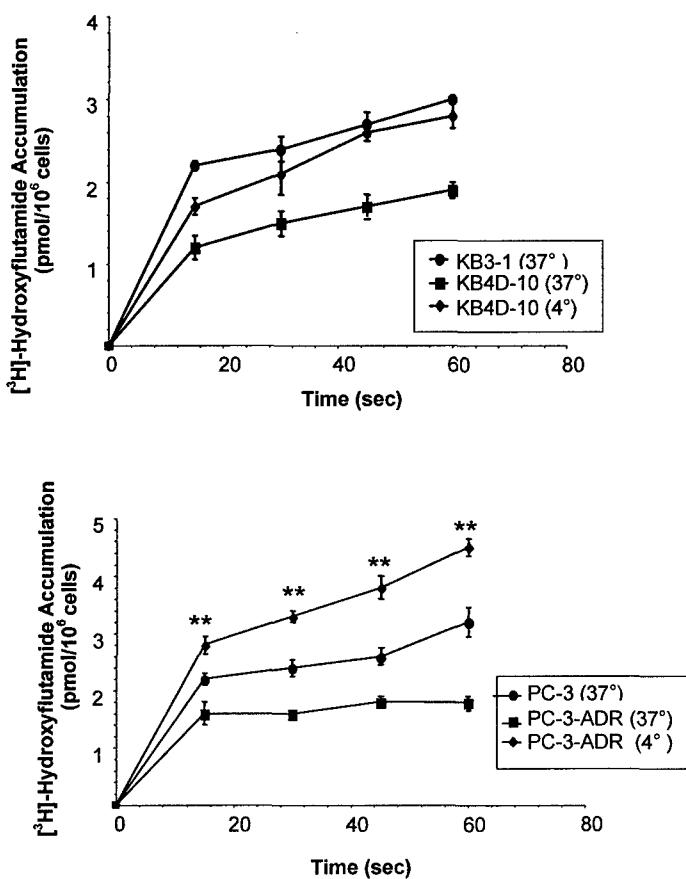


Figure 4b. Effect of temperature on hydroxyflutamide accumulation. KB3-1, KB4D-10 (top) and PC-3, PC-3-ADR (bottom) were seeded onto 24 well plates. Forty-eight hours later the cells were incubated for one hour at 4°C. The accumulation of [³H]-hydroxyflutamide was assayed as described in "Materials and Methods". Each point represents the mean \pm standard deviation of quadruplicate determinations. The results are a representative of three separate experiments (*-p<0.05, **-p<0.01, MRP1 cells treated vs. untreated).

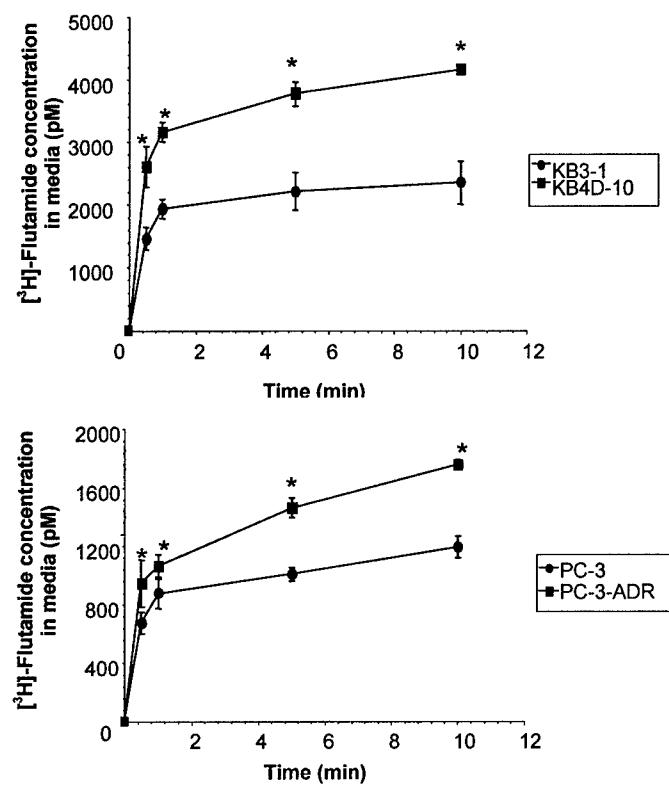


Figure 5a. Effect of MRP1 expression on efflux of flutamide. KB3-1 and KB4D-10 (top) and PC-3 and PC-3-ADR (bottom) cells were seeded onto 24 well plates, and 60 nM of [³H]-flutamide were added 48 hours later. The cells were allowed to accumulate drug for 30 minutes, then washed twice with ice-cold PBS, and resuspended in serum-free media. The appearance of [³H]-flutamide in fresh media at various time points was measured by scintillation counting. Each point represents the mean \pm standard deviation of quadruplicate determinations. The results are a representative of three separate experiments (*-p<0.05, **-p<0.01, MRP1 expressing cells vs. parental cells).

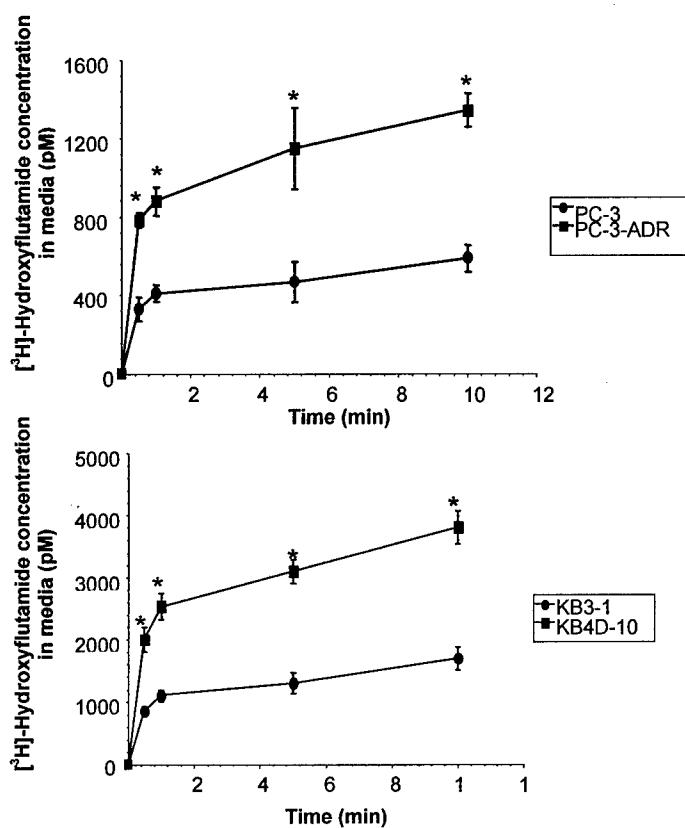


Figure 5b. Effect of MRP1 expression on efflux of hydroxyflutamide. KB3-1 and KB4D-10 (top) and PC-3 and PC-3-ADR (bottom) cells were seeded onto 24 well plates, and 60 nM of [³H]-hydroxyflutamide were added 48 hours later. The cells were allowed to accumulate drug for 30 minutes, then washed twice with ice-cold PBS, and resuspended in serum-free media. The appearance of [³H]-hydroxyflutamide in fresh media at various time points was measured by scintillation counting. Each point represents the mean \pm standard deviation of quadruplicate determinations. The results are a representative of three separate experiments (*-p<0.05, **-p<0.01, MRP1 expressing cells vs. parental cells).

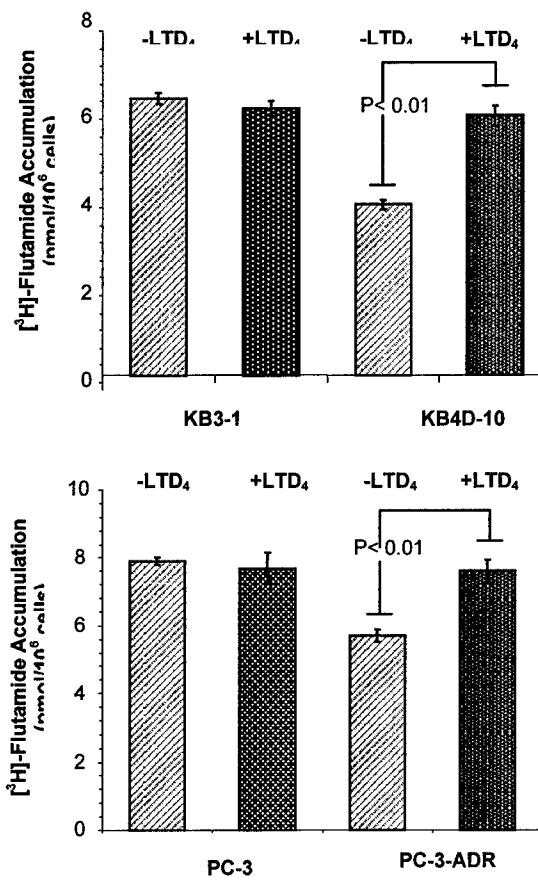


Figure 6a. Effect of leukotriene D4 on accumulation of flutamide. KB3-1 and KB4D-10 (top), and PC-3 and PC-3-ADR (bottom) were seeded onto 24 well plates and the accumulation of [³H]-flutamide was determined following a three-hour pre-incubation with 2 μ M leukotriene D4 as described in Figure 2 and “Materials and Methods”. Each point represents the mean \pm standard deviation of quadruplicate determinations. The results are a representative of three separate experiments (*- $p<0.05$, **- $p<0.01$, MRP1 cells treated vs. untreated).

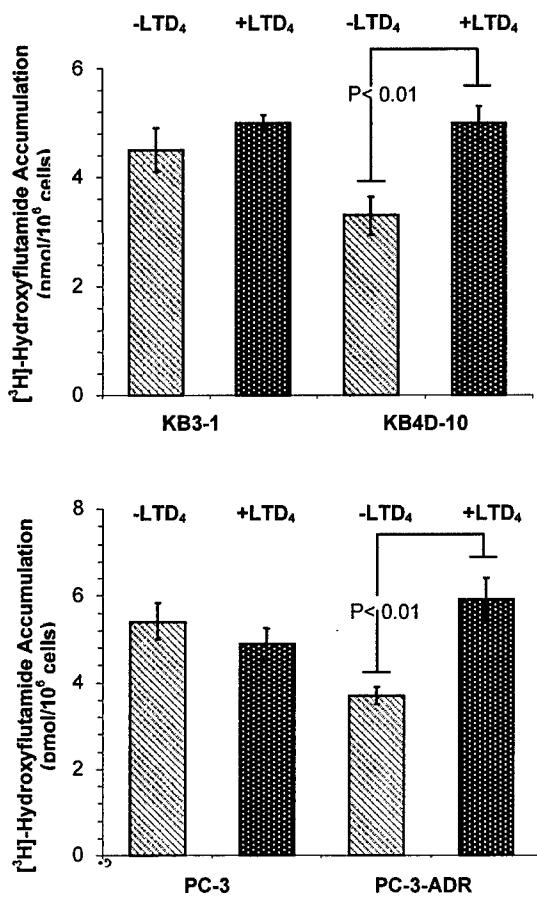


Figure 6b. Effect of leukotriene D4 on accumulation of hydroxyflutamide. KB3-1 and KB4D-10 (top), and PC-3 and PC-3-ADR (bottom) were seeded onto 24 well plates and the accumulation of [³H]-hydroxyflutamide (b) was determined following a three-hour pre-incubation with 2 μ M leukotriene D4 as described in Figure 2 and “Materials and Methods”. Each point represents the mean \pm standard deviation of quadruplicate determinations. The results are a representative of three separate experiments (*-p<0.05, **-p<0.01, MRP1 cells treated vs. untreated).

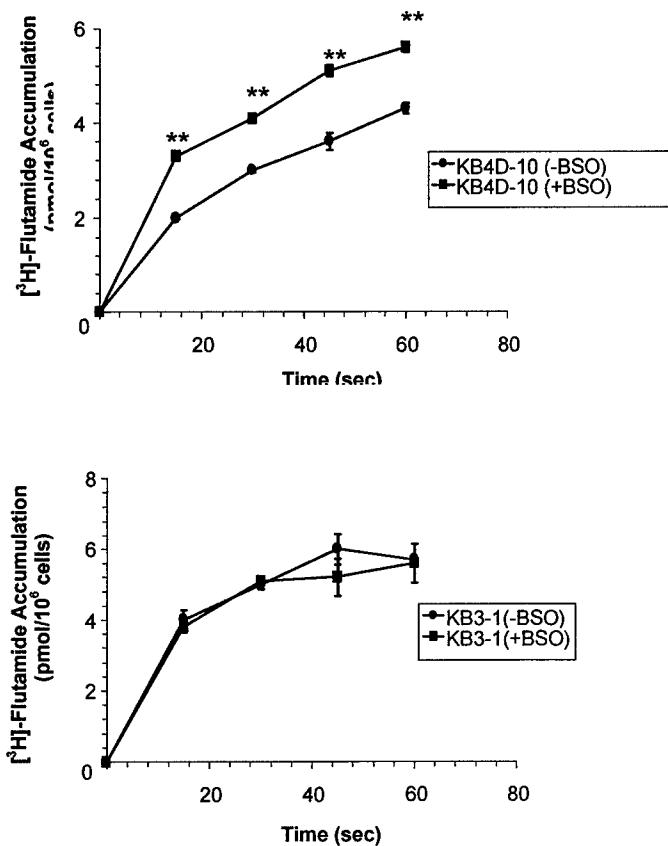


Figure 7a. Effect of glutathione depletion on the accumulation of flutamide. (A) KB3-1 (top) and KB4D-10 (bottom) cells were seeded onto 24 well plates, and 50 μ M of BSO was added 24 hours later. Drug accumulation was assayed as described in "Materials and Methods". Each point represents the mean \pm standard deviation of quadruplicate determinations. The results are a representative of three separate experiments (*-p<0.05, **-p<0.01, MRP1 cells treated vs. untreated).

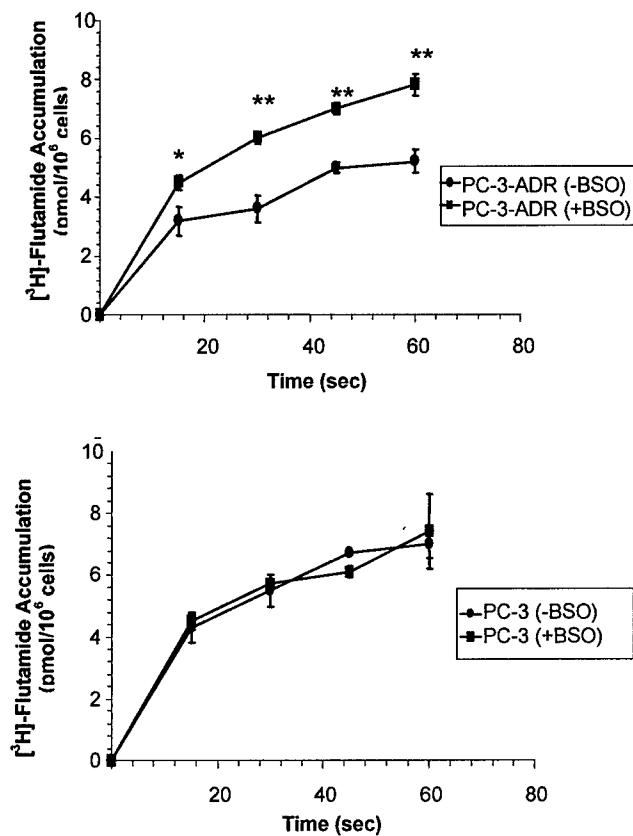


Figure 7b. Effect of glutathione depletion on the accumulation of flutamide. PC-3 (top) and PC-3-ADR (bottom) cells were seeded onto 24 well plates, and 50 μ M of BSO was added 24 hours later. Drug accumulation was assayed as described in “Materials and Methods”. Each point represents the mean \pm standard deviation of quadruplicate determinations. The results are a representative of three separate experiments (*-p<0.05, **-p<0.01, MRP1 cells treated vs. untreated).

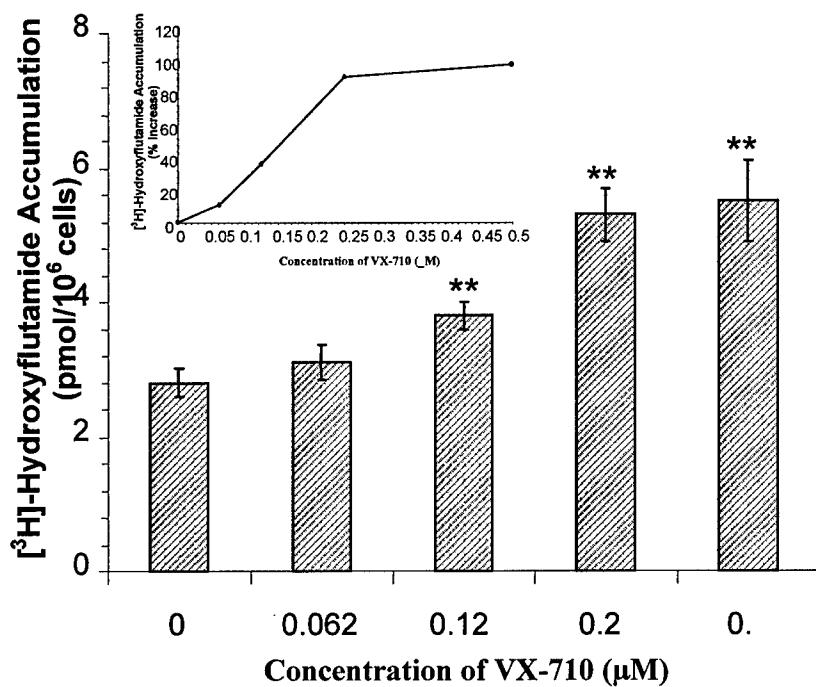


Figure 8. Effect of VX-710 on the accumulation of hydroxyflutamide. PC-3-ADR cells were plated onto 24 well plates and allowed to grow for 48 hours before VX-710 was added for 3 hours. Drug accumulation was assayed as described in Figure 4 and “Materials and Methods”. The insert signifies the percentage increase over control (no addition of VX-710). Each point represents the mean \pm standard deviation of quadruplicate determinations. The results are a representative of three separate experiments. (*-p<0.05, **-p<0.01 MRP1 cells treated vs. untreated).

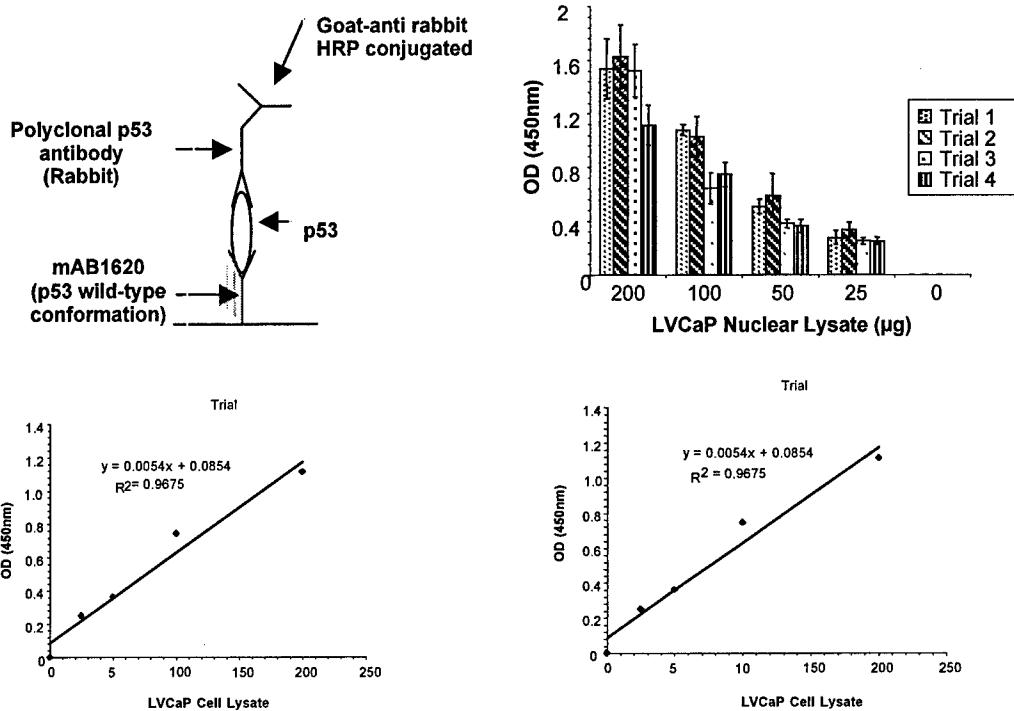


Figure 9: Standardization of p53 conformational ELISA assay. (Top left) schematic representation of conformational assay. (Top right) ELISA plates were coated with mAB1620 and blocked with 3% BSA. LVCaP nuclear lysate was added in increasing concentrations overnight at 4°C. The plates were washed one time with cold PBS and a rabbit polyclonal p53 antibody was added for one hour at room temperature. The plates were washed three times with PBS-0.05% Tween-20 and a goat anti-rabbit HRP conjugated was incubated for one hour at room temperature. The plates were washed five times with PBS-0.05% Tween-20 and the signal was developed with TMB. The experiment was repeated four times and a standard curve was established (bottom).

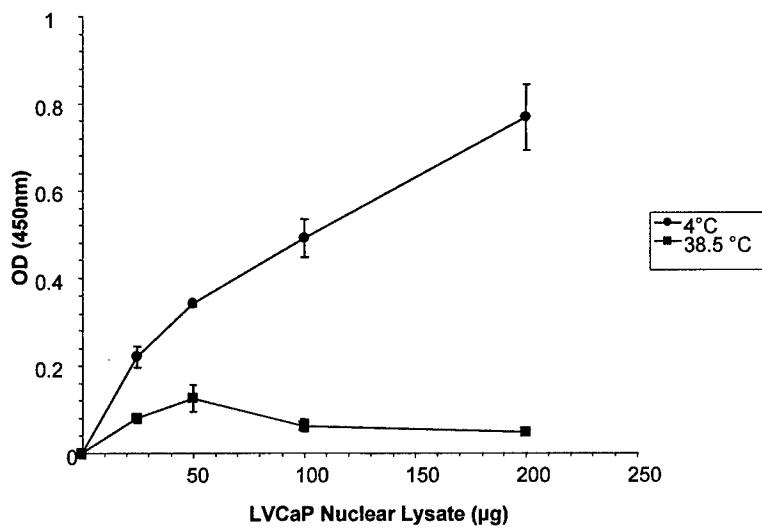


Figure 10. Effect of temperature on wild-type p53 conformation. LVCaP nuclear lysate was prepared and were heated to 38.5°C for 30 minutes prior to adding to plates coated with the wild-type antibody mAB1620. Twenty-four hours later a polyclonal p53 antibody was added for 1 hour and the signal was detected with an HRP-conjugated antibody. The results are a representative of three separate experiments.

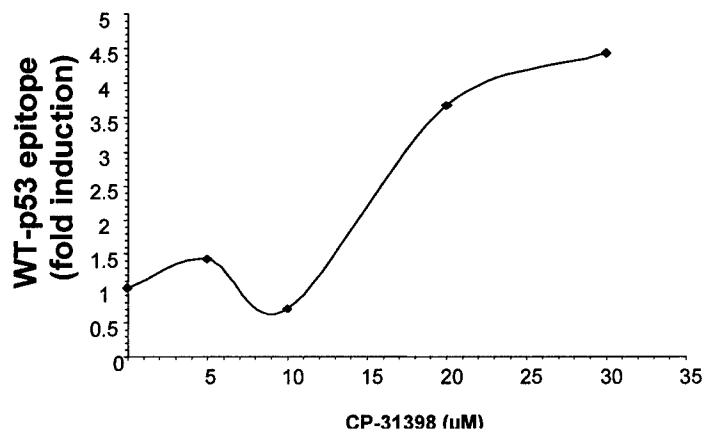


Figure 11a. Effect of CP-31398 on the Wild-Type Conformation of p53 in Mutant p53 Cells: Mutant p53 cells 10(3)-p53-175 were treated with increasing concentrations of CP-31398 for 15 hours. The cells were lysed and nuclear extracts were added to plates coated with the wild-type antibody mAB1620. Twenty-four hours later a polyclonal p53 antibody was added for 1 hour and the signal was detected with an HRP-conjugated antibody. The results are a representative of three separate experiments (*p< 0.05).

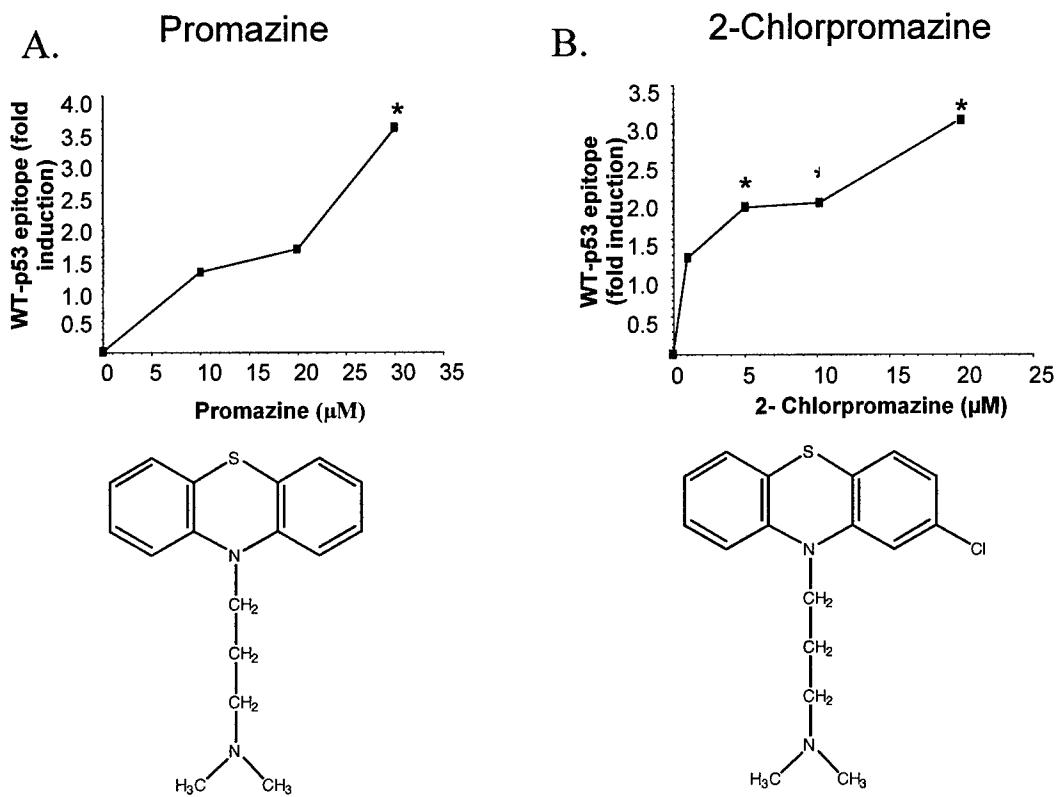


Figure 11b. Effect of Phenothiazines on the Wild-Type Conformation of p53 in Mutant p53 Cells: Mutant p53 cells 10(3)-p53-175 were treated with increasing concentrations of promazine (a) or chlorpromazine (b) 15 hours. The cells were lysed and nuclear extracts were added to plates coated with the wild-type antibody mAB1620. Twenty-four hours later a polyclonal p53 antibody was added for 1 hour and the signal was detected with an HRP-conjugated antibody. The results are a representative of three separate experiments (*p< 0.05).

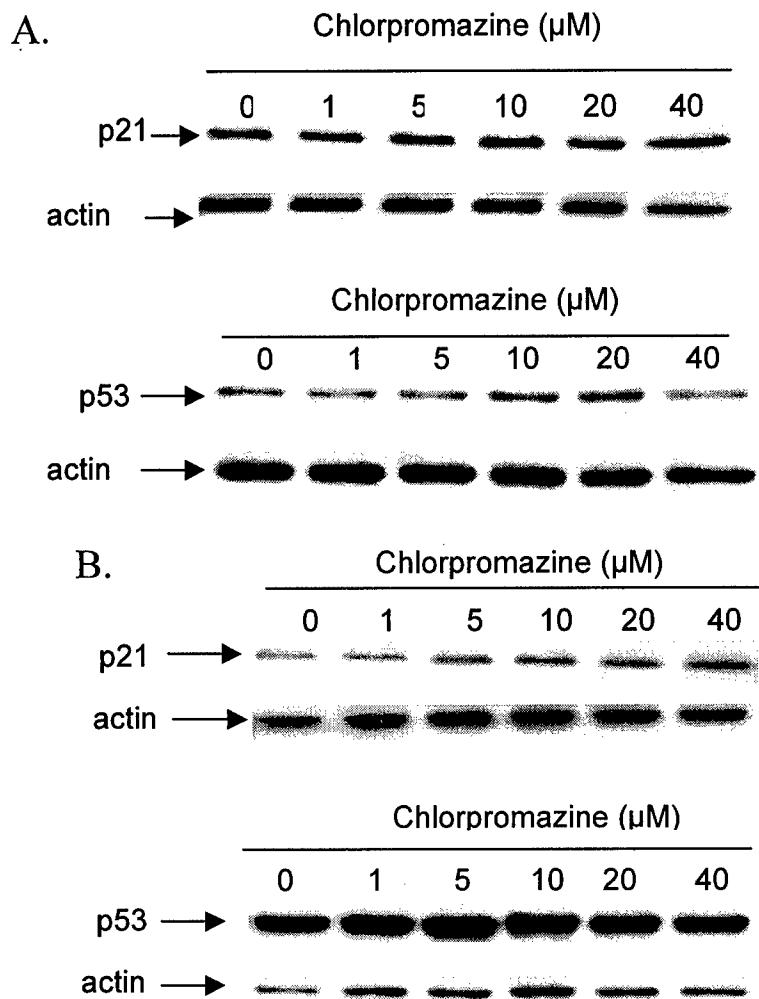


Figure 12. Effect of Chlorpromazine on p53 and p21 Expression in Prostate Cancer Cells. LNCaP (a) and LVCaP b) cells were seeded at 1×10^6 cells on 10 cm plates. Twenty-four hours later Chlorpromazine was added for 12 hours. The cells were lysed and 40 μ g of protein was loaded on 8-15% SDS-PAGE gels. The gel was transferred on a nitrocellulose membrane and probed with anti-p53 or anti-p21 antibodies. The signal was detected with an HRP-conjugated secondary antibody. The results are a representative of three separate experiments.

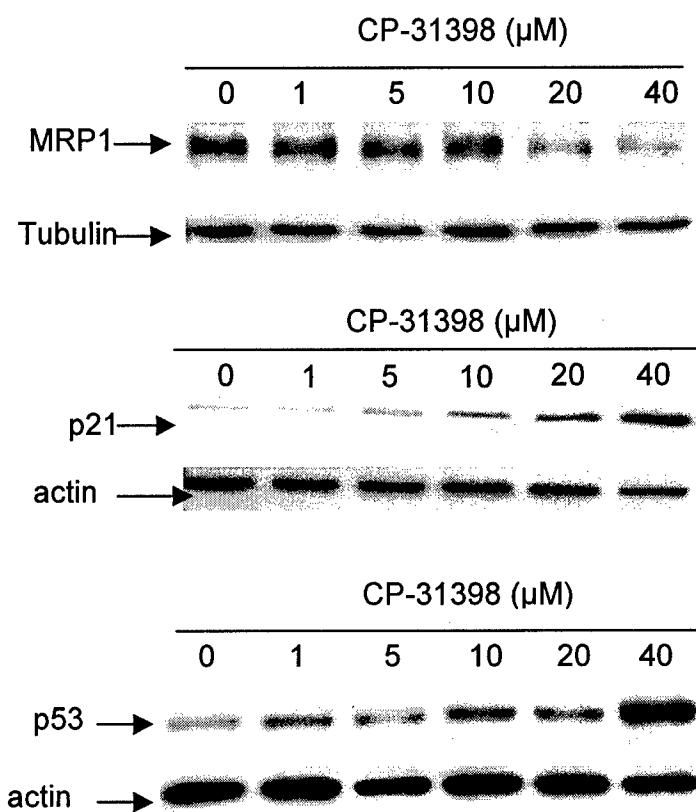


Figure 13a. Effect of CP-31398 on MRP1, p21, and p53 Expression in Prostate Cancer Cells. LNCaP cells were seeded at 1×10^6 cells on 10 cm plates. Twenty-four hours later increasing concentrations of CP-31398 was added for 12 hours. The cells were lysed and 40 μ g of protein was loaded on 6-15% SDS-PAGE gels. The gel was transferred on a nitrocellulose membrane and probed with anti-MRP1 (top), anti-p21 (middle), and anti-p53 (bottom) antibodies. The signal was detected with an HRP-conjugated secondary antibody. The results are a representative of three separate experiments.

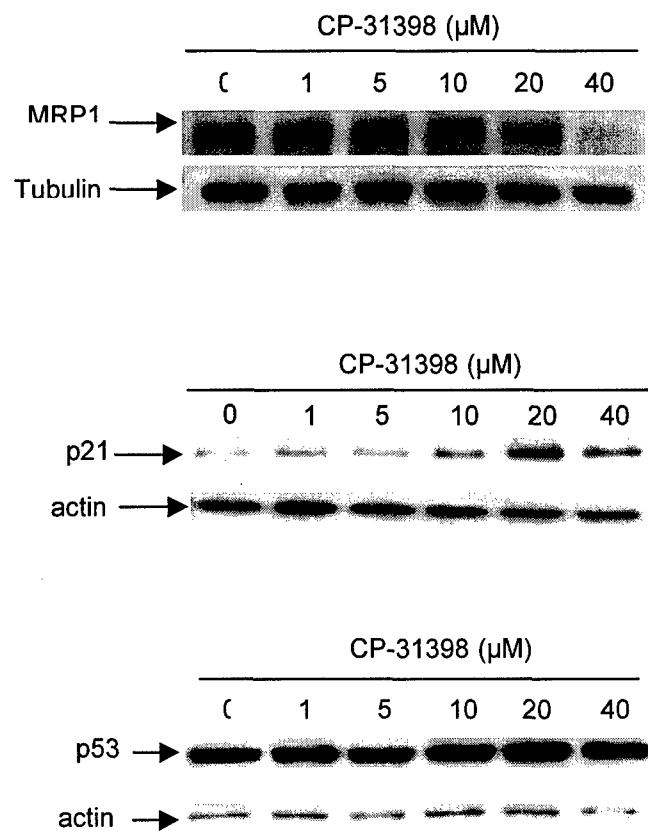


Figure 13b. Effect of CP-31398 on MRP1, p21, and p53 Expression in Prostate Cancer Cells. LVCaP cells were seeded at 1×10^6 cells on 10 cm plates. Twenty-four hours later increasing concentrations of CP-31398 was added for 12 hours. The cells were lysed and 40 μ g of protein was loaded on 6-15% SDS-PAGE gels. The gel was transferred on a nitrocellulose membrane and probed with anti-MRP1 (top), anti-p21 (middle), and anti-p53 (bottom) antibodies. The signal was detected with an HRP-conjugated secondary antibody. The results are a representative of three separate experiments.

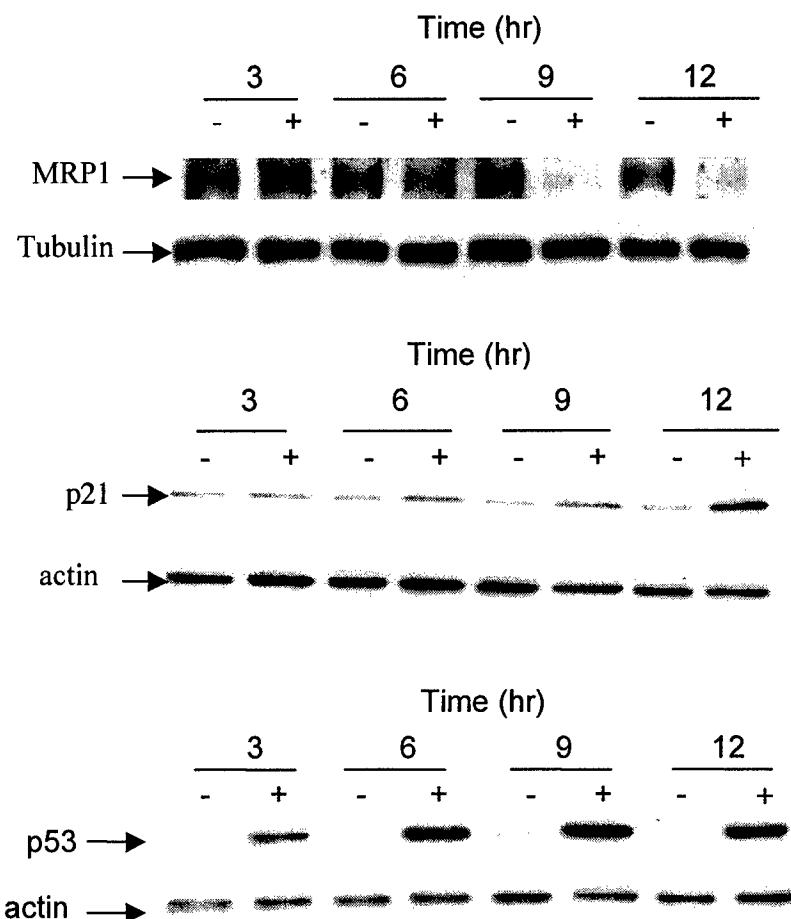


Figure 14a. Effect of CP-31398 on MRP1 and p21 Expression in Prostate Cancer Cells. LNCaP cells were seeded at 1×10^6 cells on 10 cm plates. Twenty-four hours later the cells were incubated with (+) or without (-) 40 μ M of CP-31398 for different time intervals. The cells were lysed and 40 μ g of protein was loaded on 6-15% SDS-PAGE gels. The gel was transferred on a nitrocellulose membrane and probed with anti-MRP1 (top) or anti-p21 (middle) or anti-p53 (bottom) antibodies. The signal was detected with an HRP-conjugated secondary antibody. The results are a representative of three separate experiments.

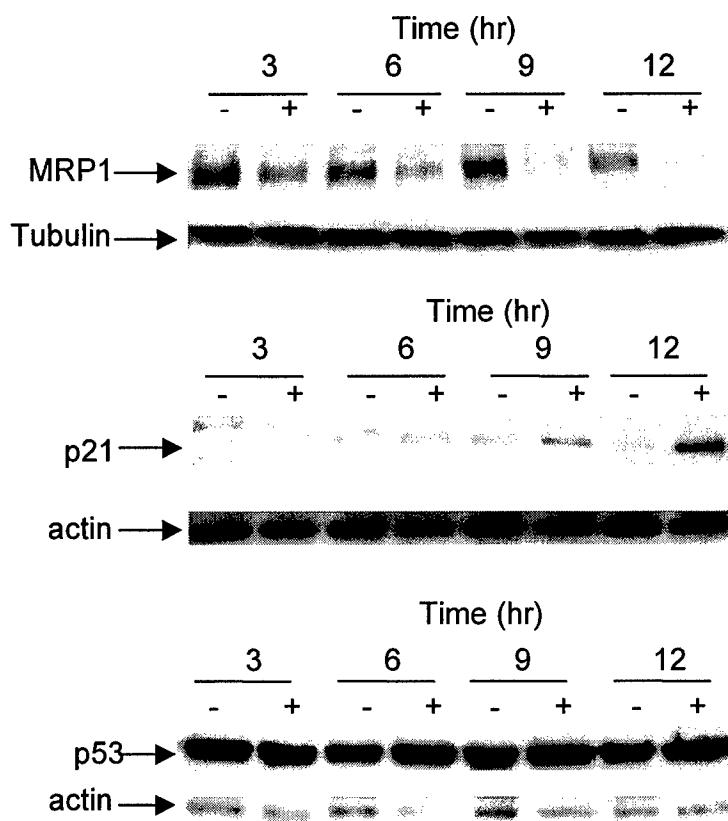


Figure 14b. Effect of CP-31398 on MRP1 and p21 Expression in Prostate Cancer Cells. LVCaP cells were seeded at 1×10^6 cells on 10 cm plates. Twenty-four hours later the cells were incubated with (+) or without (-) 40 μM of CP-31398 for different time intervals. The cells were lysed and 40 μg of protein was loaded on 6-15% SDS-PAGE gels. The gel was transferred on a nitrocellulose membrane and probed with anti-MRP1 (top) or anti-p21 (middle) or anti-p53 (bottom) antibodies. The signal was detected with an HRP-conjugated secondary antibody. The results are a representative of three separate experiments.

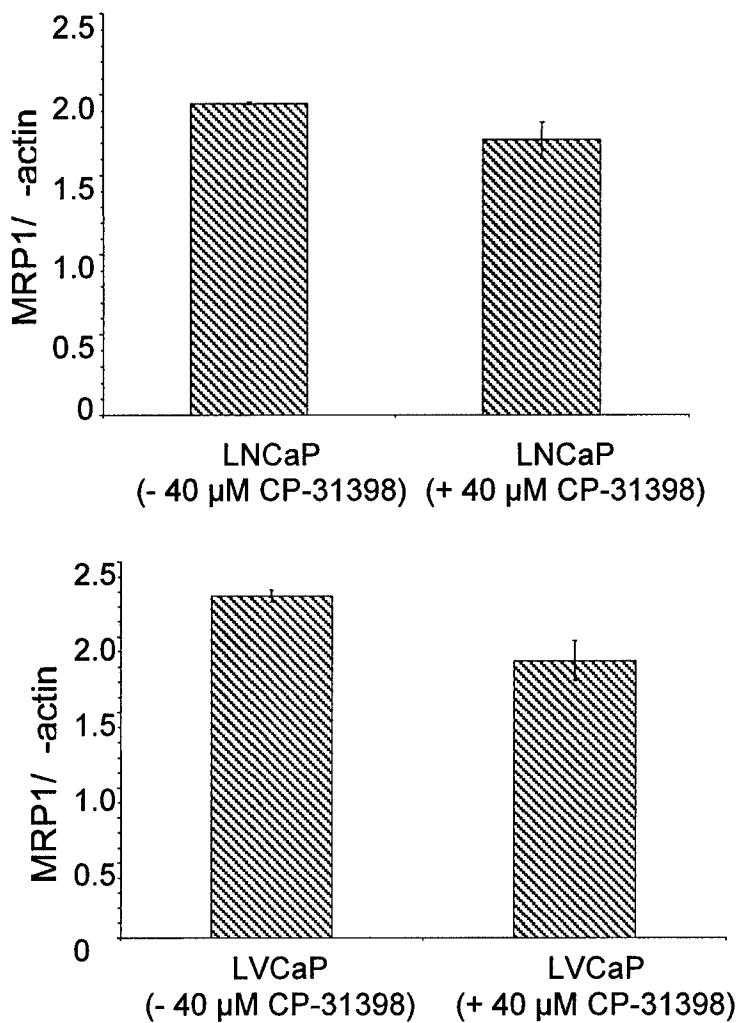


Figure 15. Effect of CP-31398 on MRP1 mRNA. LNCaP (top) and LVCaP (bottom) were treated with 40 μ M of CP-31398 for 12 hours and mRNA was isolated using trizol. One microgram of RNA was used to create single strand DNA by reverse transcription. The cDNA was PCR amplified and the product was visualized on a 1% agarose gel and stained with ethidium bromide. MRP1 expression was normalized to actin. The results are a representative of three separate experiments.

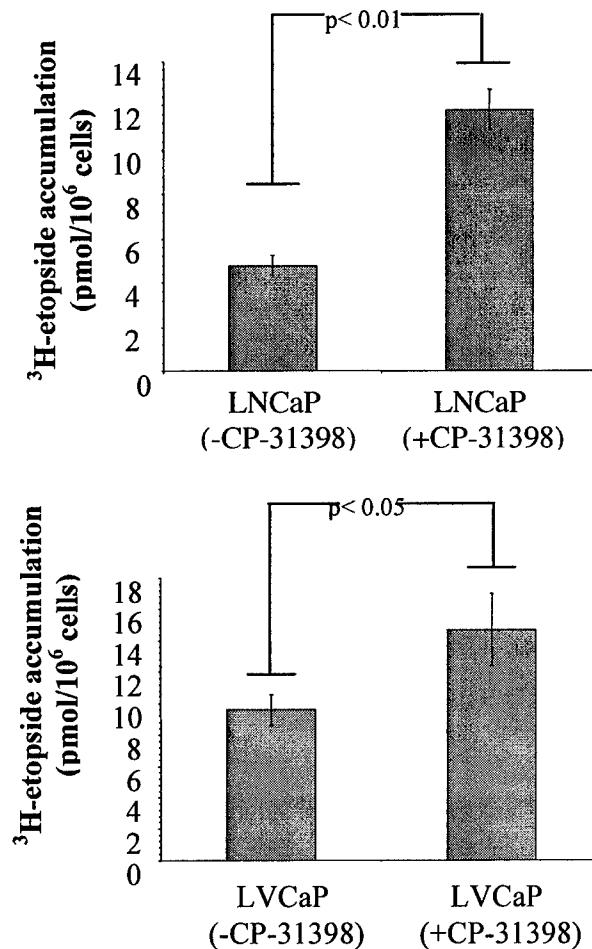


Figure 16a. Effect of CP-31398 on the Accumulation of Etoposide. LNCaP (top) and LVCaP (bottom) cells were plated at 5×10^5 cells on 60 mm plates and 40 μM of CP-31398 was added 24 hours later. Twelve hours later the cells were trypsinized and added to 1.5 mL microfuge tubes. After washing once with serum free media 5 μM ${}^3\text{H}$ -etoposide was added for two hours. The cells were washed with ice cold-PBS, solubilized with 1% SDS, and the radioactivity was measured by scintillation counting. Each point represents the mean \pm standard deviation of quadruplicate determinations. The results are a representative of three separate experiments.

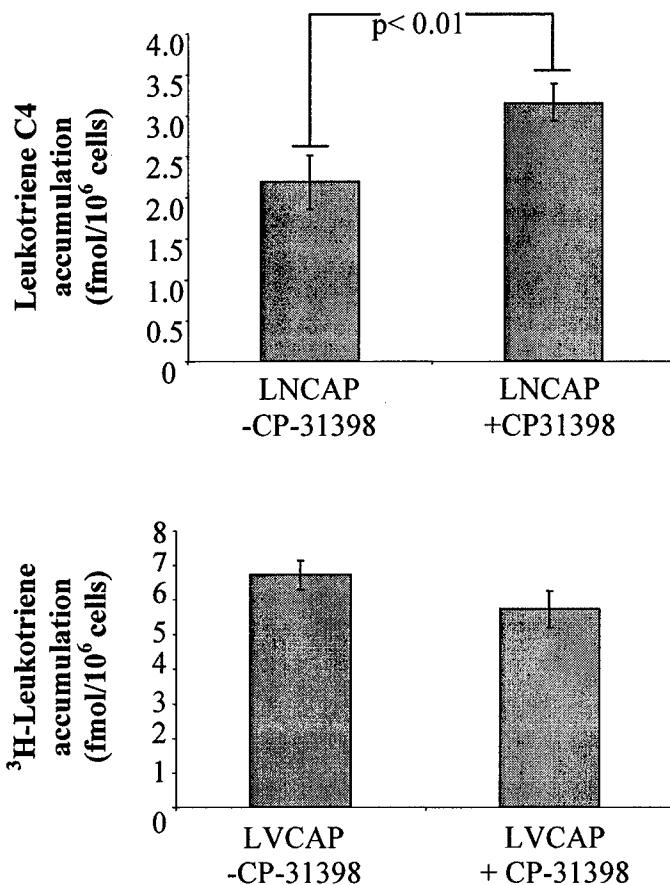


Figure 16b. The Effect of CP-31398 on the Accumulation of Leukotriene C4. LNCaP (top) and LVCaP (bottom) cells were plated at 5x10⁵ cells on 60 mm plates and 40 μ M of CP-31398 was added 24 hours later. Twelve hours later the cells were trypsinized and added to 1.5 mL microfuge tubes. After washing once with serum free media 1nM ³H-leukotriene C4 was added for two hours. The cells were washed with ice cold-PBS, solubilized with 1% SDS, and the radioactivity was measured by scintillation counting. Each point represents the mean \pm standard deviation of quadruplicate determinations. The results are a representative of three separate experiments.

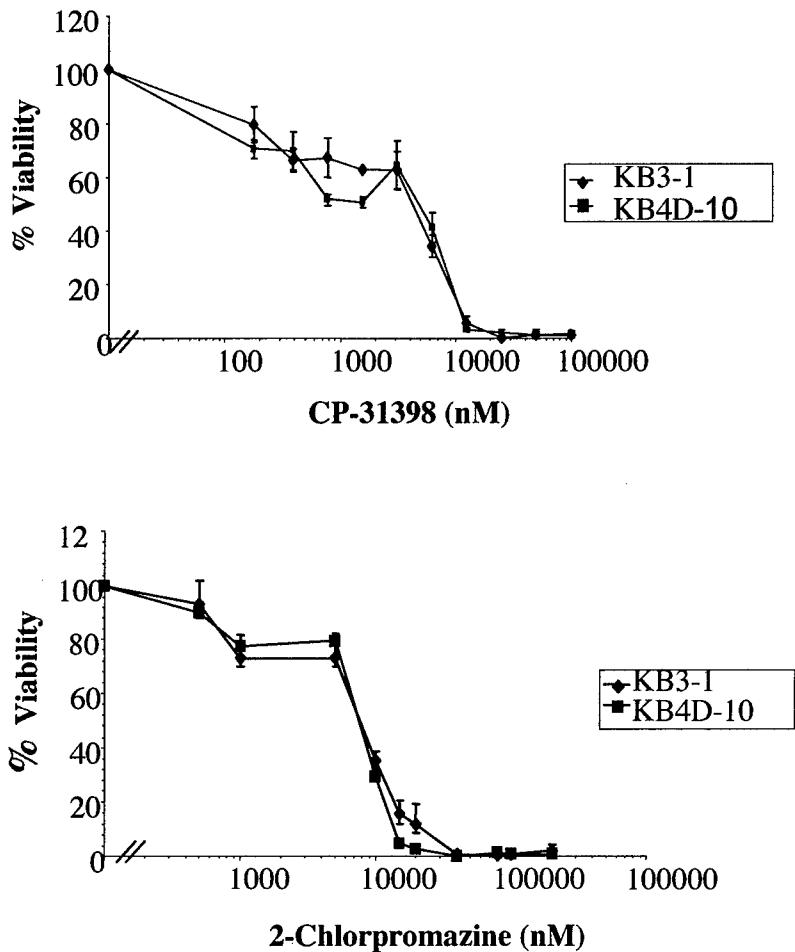


Figure 17. Effect of MRP1 on Sensitivity of Potential p53 Stabilizing Compounds. KB3-1 and KB4D-10 were plated at a density of $2.0-4.0 \times 10^3$ /well in a 96 well plate. Twenty-four hours later CP-31398 (top) and Chlorpromazine (bottom) were added at increasing concentrations. Forty-eight hours later cell viability was determined by adding 2mg/mL of MTT for three hours. The results are a representative of three separate experiments.

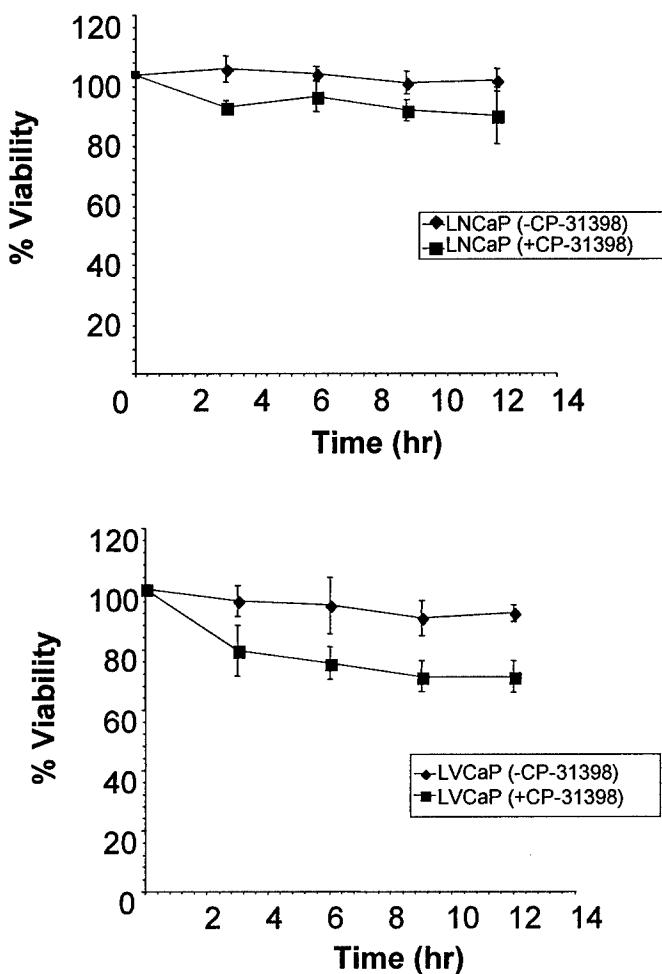


Figure 18. Effect of CP-31398 on Cell Viability. LNCaP (top) and LVCaP (bottom) were plated at a density of 5×10^5 in 60 mm plates. Twenty four hours later 40 µM of CP-31398 was added at different time intervals. The cells were trypsinized and cell viability was measured by trypan blue exclusion. The results are a representative of three separate experiments.

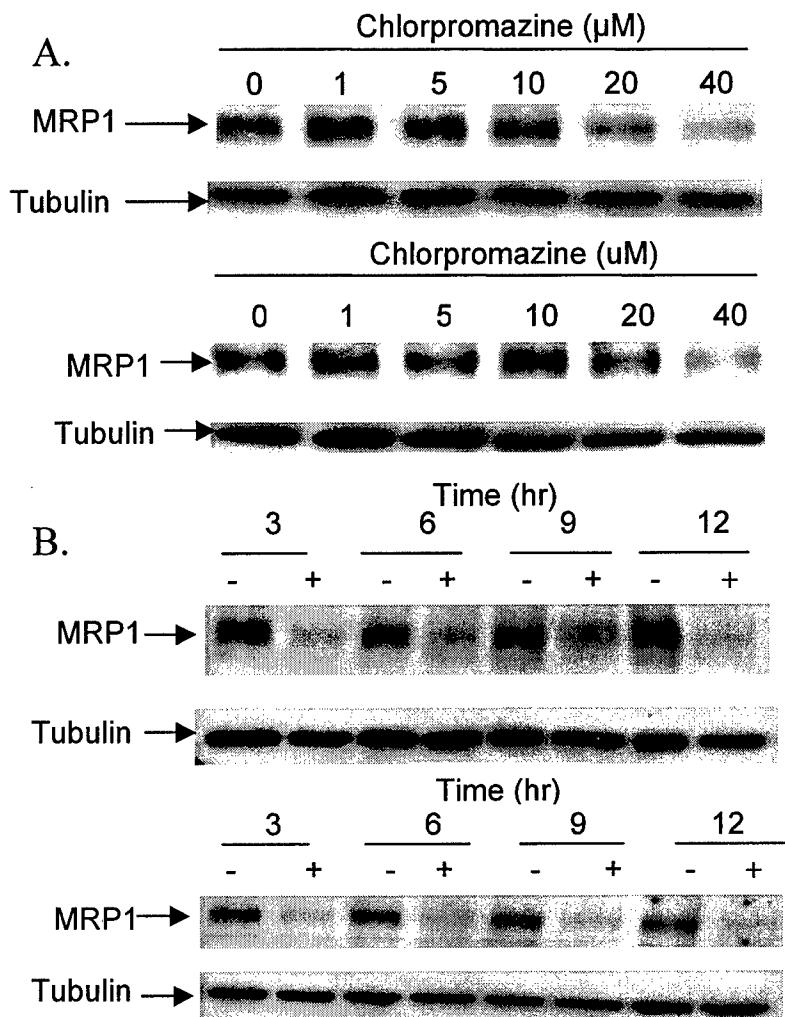


Figure 19. Effect of Chlorpromazine on MRP1 Expression in Prostate Cancer Cells. LNCaP (top) and LVCaP (bottom) cells were seeded at 1×10^6 cells on 10 cm plates. Twenty-four hours later increasing concentrations of chlorpromazine was added for 12 hours (a) or incubated with (+) or without (-) 40 μ M of CP-31398 for different time intervals (b). The cells were lysed and 40 μ g of protein was loaded on 6% SDS-PAGE gels. The gel was transferred on a nitrocellulose membrane and probed with anti-MRP1 antibodies. The signal was detected with an HRP-conjugated secondary antibody. The results are a representative of three separate experiments.

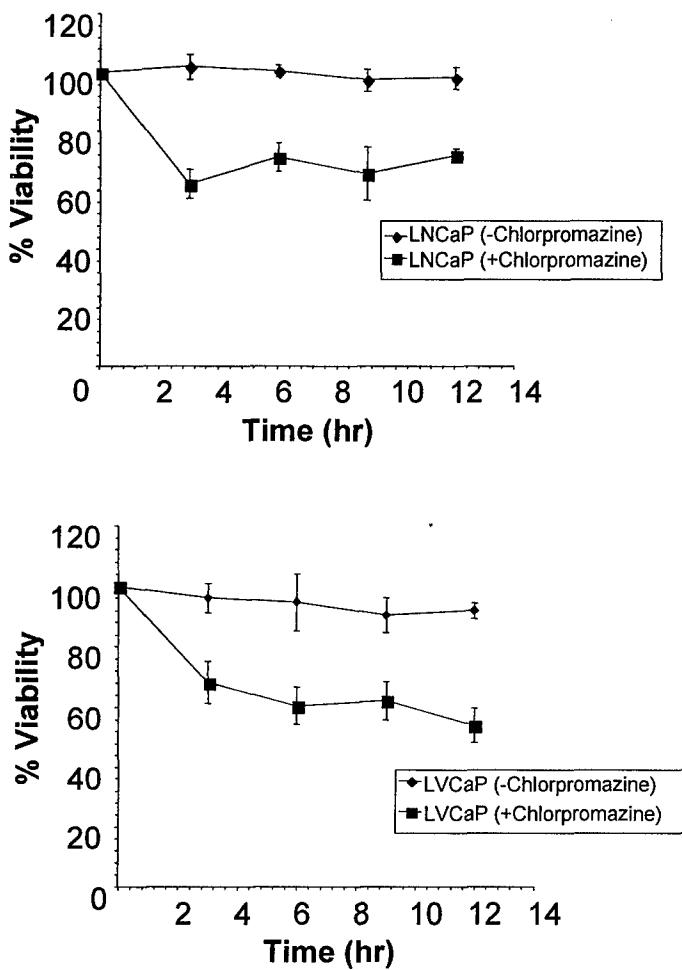


Figure 20. Effect of Chlorpromazine on Cell Viability. LNCaP (top) and LVCaP (bottom) were plated at a density of 5×10^5 in 60 mm plates. Twenty four hours later 40 μM of CP-31398 was added at different time intervals. The cells were trypsinized and cell viability was measured by trypan blue exclusion. The results are a representative of three separate experiments.

Effect of the Multidrug Resistance Protein on the Transport of the Antiandrogen Flutamide¹

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ABSTRACT

Prostate cancer is the most common noncutaneous malignancy of American men. Although it can be initially treated with androgen deprivation therapy, tumors that relapse become resistant to future hormonal manipulation. We previously found that the multidrug resistance protein (MRP), MRP1, is overexpressed in advanced stage and grade human prostate cancer and is negatively regulated by p53. In this study, we sought to determine whether the cellular accumulation of the antiandrogen flutamide, a drug commonly used in the treatment of prostate cancer, is affected by MRP1 expression. There were significant differences between the wild-type and MRP1-overexpressing cells in efflux and accumulation of flutamide and hydroxyflutamide, its active metabolite. In contrast, transport of dihydrotestosterone was not affected by MRP1. Treating the cells with leukotriene D4, a known MRP1 substrate, or VX-710, an MRP1 modulator, restored flutamide and hydroxyflutamide accumulation. Finally, intracellular glutathione depletion with buthionine sulfoximine or energy depletion using 2-deoxy-D-glucose/sodium azide restored flutamide accumulation to that of parental cells while incubating the cells at 4°C abolished MRP1-mediated transport. In summary, these studies indicate that flutamide and hydroxyflutamide but not dihydrotestosterone are transported by MRP1 and that these findings may contribute to our understanding of resistance to hormone refractory prostate cancer.

INTRODUCTION

Prostate cancer is the most common noncutaneous malignancy in American men (1). When diagnosed at an early stage, *i.e.*, before cancer cells escape the capsule of the prostate, treatment with surgery or radiation can produce a 5-year survival of 95% (1). When prostate cancer recurs or is diagnosed at an advanced stage, standard treatment includes pharmacological or surgical castration (2, 3).

Most prostate cancers require androgens for growth and development. Flutamide is a nonsteroidal antiandrogen that acts as a competitive inhibitor of dihydrotestosterone for the androgen receptor (2) and is often used as a part of initial treatment. Flutamide and its active metabolite hydroxyflutamide block expression of genes with promoters that contain androgen response elements and also prevent androgen-dependent stabilization of the androgen receptor (4). Although >50% of patients initially respond to androgen deprivation, most patients relapse within a median of 12–18 months, at which time, they are resistant to additional hormonal therapy (5). Retreatment with antiandrogen therapy or chemotherapy of patients with hormone-refractory disease has not been shown to significantly improve the overall survival of 2–3 years (1).

Several factors may contribute to resistance after relapse from androgen deprivation therapy. These include increased affinity of the receptor for testosterone because of mutation of the androgen receptor

(6, 7), increased androgen receptor-mediated transcription through activation of cyclic AMP-dependent protein kinase A (8), and decreased apoptosis attributable to mutations in p53 (9).

We recently found that progression from benign prostate epithelium to high-grade prostate cancer correlated with the expression of drug resistance proteins (10). Specifically, our results indicated that overexpression of MRP³¹ correlated with expression of mutant p53 (10) and that the expression of MRP1 was repressed by the wild-type protein (11).

MRP1 is a M_r 190,000 membrane protein belonging to the ABC family of transporters. MRP1 pumps negatively charged substrates from the cytosol to the extracellular environment (12–14). Cotransport with glutathione (*e.g.*, doxorubicin and vincristine; Refs. 15, 16) or conjugation with glutathione, glucuronides, or sulfates (*e.g.*, leukotrienes) can enhance transport of MRP1 substrates (16, 17).

The structural features of drugs that are transported by ABC transporters have been extensively studied (18, 19). We noted that the antiandrogen flutamide shares several of these characteristics, including a weak negative charge and an electron-withdrawing trifluoro group attached to a hydrophobic ring (Refs. 18, 19; Fig. 1). Therefore, the purpose of this study was to determine if MRP1 expression affected the transport of flutamide in human cancer cells.

MATERIALS AND METHODS

Drugs. Leukotriene D4, buthionine sulfoximine, sodium azide, and 2-deoxy-D-glucose were obtained from Sigma Chemicals (St. Louis, MO). [³H]Flutamide (10–13 Ci/mmol) and [³H]hydroxyflutamide (9 Ci/mmol) were obtained from Moravek Biochemicals (Brea, CA). 5 α -Dihydro[1 α , 2 α (n)-³H] testosterone was obtained from Amersham Pharmacia (Bedminster, NJ). The MRP1 primary antibody, QCRL-1, and P-glycoprotein antibody, C219, were obtained from Signet Laboratories (Dedham, MA). The goat antimouse secondary antibody was obtained from Dako (Carpentina, CA). The MRP6 antibody was obtained through Santa Cruz Biotechnology (Santa Cruz, CA). The MRP1 modulator VX-710 was a gift from Vertex Pharmaceuticals (Cambridge, MA).

Cell Lines and Culture Conditions. The parental KB3-1 and the MRP1-expressing cell line KB4D-10 (a gift from Vertex Pharmaceuticals) were grown in DMEM with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin. KB4D-10 cells were transfected with an MRP1 expression vector containing a full-length cDNA and grown in the same conditions as its parental cell line, except that the media contained 10 ng/ml doxorubicin. PC-3, the MRP1-overexpressing cell line PC-3-ADR (a gift from Dr. William Nelson, John Hopkins, Baltimore, MD), and the P-glycoprotein overexpressing cell line MCF-7-ADR were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin. PC-3-ADR cells were selected for MRP1 by adding increasing amounts of doxorubicin and selecting viable colonies; these cells were maintained in 100 nM doxorubicin. All cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂/95% air. Cell lines were free of *Mycoplasma* and fungi and were discarded after 3 months; new cell lines were obtained from frozen stocks.

Western Analysis. Cell lysates were prepared, and protein concentrations determined as previously described (11) with slight modifications. Briefly,

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³ The abbreviations used are: MRP, multidrug resistance protein; BSO, buthionine sulfoximine; PBS, Phosphate Buffered Saline; ABC, ATP binding cassette.

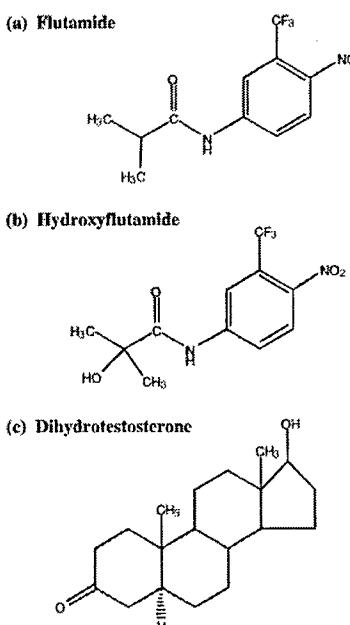


Fig. 1. Structure of flutamide (a), its active metabolite hydroxyflutamide (b), and dihydrotestosterone (c).

cells were lysed with radioimmunoprecipitation assay buffer [10 mM sodium phosphate (pH 7.2), 1% NP40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, and 2 mM EDTA, supplemented with fresh 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 50 μ g/ml leupeptin], and the supernatant removed after centrifugation at 16,000 \times g for 30 min. The supernatant was sonicated using a Virsonic 100 sonicator at 3 W. Fifty μ g of protein were separated on an 8% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were blocked with 5% milk in PBS with 0.05% Tween 20, washed, and incubated with the MRP1 antibody QCRL-1 overnight. Binding of the primary antibody was detected using a goat antimouse horseradish peroxidase-conjugated secondary antibody. Binding was detected using the enhanced chemiluminescence detection system (Amersham Pharmacia) as described in the manufacturer's protocol.

Drug Accumulation, Uptake, and Efflux. Drug accumulation and uptake were determined by seeding cells (5.0×10^4 - 1.0×10^5 cells/ml/well) onto 24-well plates. After 48 h, cells were washed with serum-free media containing 25 mM HEPES and incubated with 60 nM [3 H]flutamide, 60 nM [3 H]hydroxyflutamide, or 20 nM 5 α -dihydro[1 α , 2 α (n)- 3 H]testosterone for varying periods of time. Additional experiments were performed by incubating the cells at 4°C for 1 h or using glucose-free media supplemented with 15 mM sodium azide and 50 mM 2-deoxy-D-glucose for 30 min before drug incubation. The reactions were stopped by adding ice-cold PBS, and the cells were lysed immediately with 1% SDS. For measuring drug efflux, cells (5.0×10^4 - 1.0×10^5 cells/ml/well) were seeded onto 24-well plates, grown for 48 h, and washed with serum-free media. The cells were then incubated with [3 H]flutamide or [3 H]hydroxyflutamide for 30 min and washed twice with ice-cold PBS. Two hundred and fifty μ l of serum-free media were added to each well and incubated for varying periods of time. The appearance of drug in fresh media in separate wells was measured over time by liquid scintillation counting.

RESULTS

MRP1 Expression in Cell Lines. To determine the effect of MRP1 overexpression on flutamide accumulation, we used cell lines that overexpress MRP1 either by transfection or selection. Fig. 2 confirms the overexpression of MRP1 as compared with the parental controls and the lack of detectable P-glycoprotein in the drug-resistant cell lines.

Effect of MRP1 on Transport of Flutamide. To assess whether overexpression of MRP1 changes flutamide accumulation, we com-

pared the accumulation of [3 H]flutamide, [3 H]hydroxyflutamide, and [3 H]dihydrotestosterone in MRP1-overexpressing cells to that of parental cell lines. Fig. 3 demonstrates that KB4D-10 and PC-3-ADR cells accumulated significantly less [3 H]flutamide (Fig. 3A) and [3 H]hydroxyflutamide (Fig. 3B) than the parental controls. This difference was observed after 15 s of incubation; steady-state accumulation was achieved after 1 min (Fig. 3 and Table 1). In contrast, the accumulation of [3 H]dihydrotestosterone was not affected by MRP1 expression (Fig. 3C and Table 1).

To verify that the decrease in flutamide accumulation in MRP1 cells was energy dependent, we preincubated cells with sodium azide and 2-deoxy-D-glucose for 30 min. Under these conditions, steady-state accumulation of flutamide and hydroxyflutamide was restored to that of the parental cells (Fig. 4). In addition, incubation of the cells at 4°C abolished the MRP1-mediated flutamide transport (Fig. 5). In both instances, these conditions had no effect on accumulation of flutamide and hydroxyflutamide in sensitive cells (data not shown).

We next asked whether the difference observed in steady-state drug accumulation in MRP1 cells was attributable to changes in drug efflux. Fig. 6 shows that the efflux of [3 H]flutamide (Fig. 6A) and [3 H]hydroxyflutamide (Fig. 6B) into drug-free media was significantly increased in cell lines overexpressing MRP1.

Leukotriene D4 enters cells passively (20), is transported by MRP1, and can competitively block the transport of MRP1 substrates (21). To test whether leukotriene D4 could compete for [3 H]flutamide and [3 H]hydroxyflutamide transport, cell lines were incubated with the antiandrogens after a 3-h preincubation with leukotriene D4. Fig. 7 demonstrates that leukotriene D4 restored the accumulation of flutamide and hydroxyflutamide in cell lines overexpressing MRP1 but had no effect on the accumulation of the antiandrogens in parental cells.

Glutathione enhances transport of MRP substrates by either direct conjugation or cotransport (13, 22). To test the effects of glutathione on flutamide transport, we depleted cellular glutathione with BSO and measured the effect on flutamide accumulation. As shown in Fig. 8, BSO increased the accumulation of flutamide in MRP1-overexpressing cell lines but had no effect on parental cells.

To determine if VX-710, an inhibitor of MRP1 (23, 24), affected the transport of the clinically active agent hydroxyflutamide, we studied its effect on hydroxyflutamide accumulation. Fig. 9 demonstrates that VX-710 increased the accumulation of hydroxyflutamide in a dose-dependent manner. There was no effect of the drug on the transport of hydroxyflutamide in wild-type cells (data not shown).

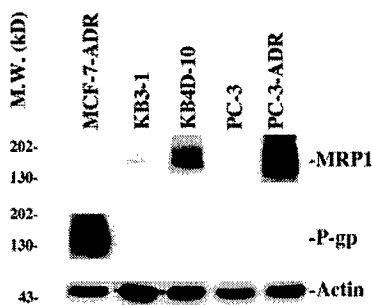


Fig. 2. Expression of MRP1 in KB4D-10 and PC-3-ADR cancer cell lines. Fifty μ g of cell lysate from each cell line were resolved on an 8% SDS-PAGE and probed with antibodies to MRP1 (QCRL-1; Signet Laboratories), β -actin (Sigma Chemicals) or P-glycoprotein (Signet Laboratories) as described in "Materials and Methods." The results are a representative of three separate experiments.

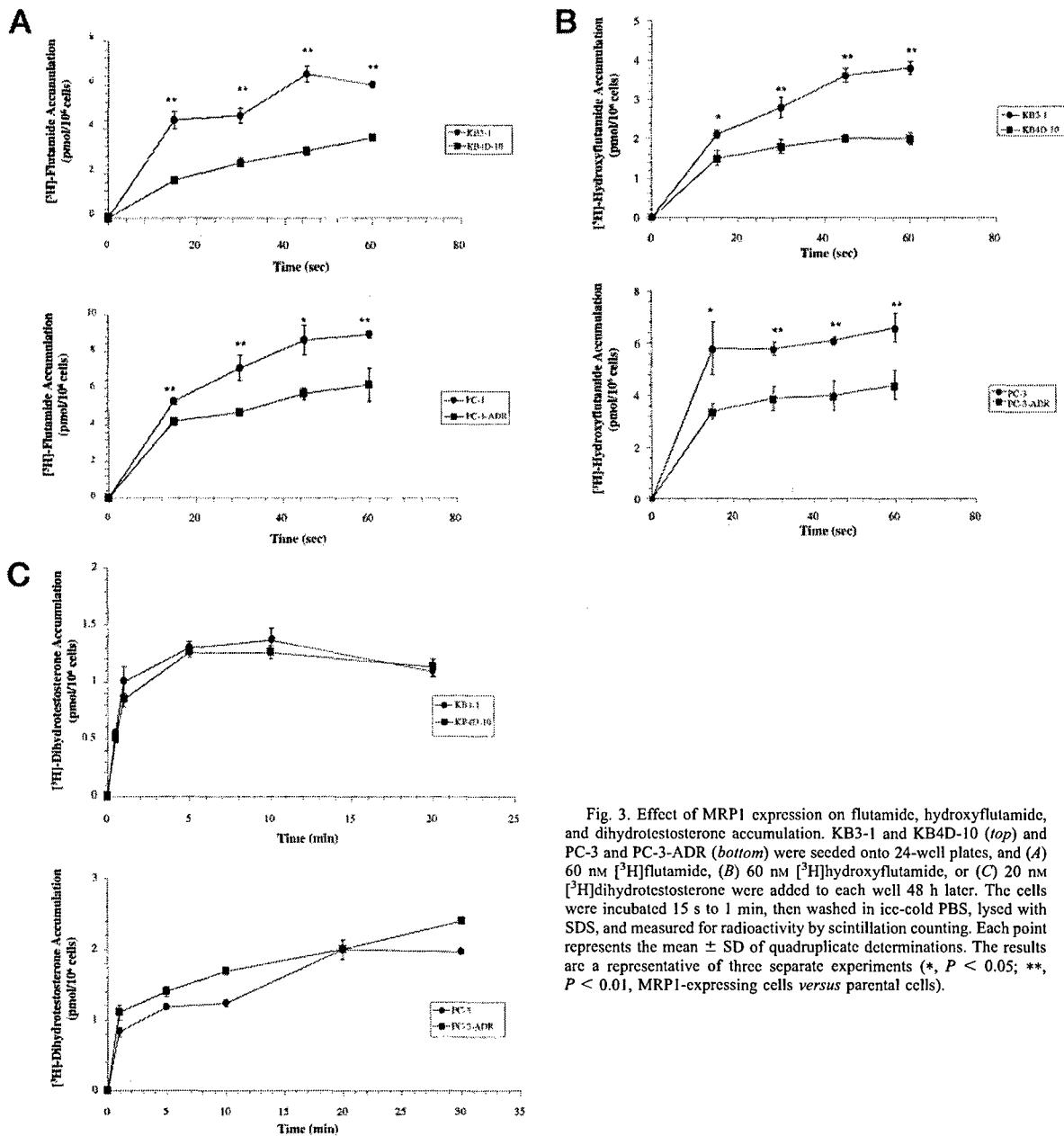


Fig. 3. Effect of MRP1 expression on flutamide, hydroxyflutamide, and dihydrotestosterone accumulation. KB3-1 and KB4D-10 (*top*) and PC-3 and PC-3-ADR (*bottom*) were seeded onto 24-well plates, and (*A*) 60 nm [³H]flutamide, (*B*) 60 nm [³H]hydroxyflutamide, or (*C*) 20 nm [³H]dihydrotestosterone were added to each well 48 h later. The cells were incubated 15 s to 1 min, then washed in ice-cold PBS, lysed with SDS, and measured for radioactivity by scintillation counting. Each point represents the mean \pm SD of quadruplicate determinations. The results are a representative of three separate experiments (*, $P < 0.05$; **, $P < 0.01$, MRP1-expressing cells versus parental cells).

DISCUSSION

Resistance to antiandrogen therapy is a hallmark of advanced prostate cancer (1). We had several reasons to suspect that resistance to the antiandrogen flutamide might be mediated by MRP1. First, we had previously shown that MRP1 expression increased with advanced disease (10). Second, the structure of flutamide resembled other compounds transported by MRP1 and P-glycoprotein (18). In this study, we provide evidence for the first time that the antiandrogens flutamide and hydroxyflutamide are transported by MRP1.

Steady-state accumulation of flutamide and hydroxyflutamide is decreased (Fig. 3), and efflux is increased (Fig. 6) in cells that overexpress MRP1. To rule out that the rapid alterations in transport represented membrane binding, cells were pretreated with sodium azide and 2-deoxy-D-glucose or incubated at 4°C. Energy depletion or incubation at 4°C abolished the differences in drug accumulation between sensitive and resistant cells, consistent with the energy dependence of MRP-mediated transport. Steady state was achieved

rapidly; the accumulation of flutamide and hydroxyflutamide was the same at 30 min as at 1 min (Table 1). To verify that the effect was not transient, we incubated the cells up to 6 h and saw a similar observation (data not shown).

Table 1 Steady-state accumulation of flutamide, hydroxyflutamide, and dihydrotestosterone in sensitive and MRP1 cell lines^a

Cell line	Flutamide (pmol/10 ⁶ cells)	Hydroxyflutamide (pmol/10 ⁶ cells)	Dihydrotestosterone (pmol/10 ⁶ cells)
PC-3	7.4 \pm 0.3 ^b	4.6 \pm 0.6 ^b	1.1 \pm 0.1
PC-3-ADR	5.9 \pm 0.8	3.1 \pm 0.6	1.2 \pm 0.2
KB3-1	6.9 \pm 0.9 ^c	5.2 \pm 0.6 ^b	1.4 \pm 0.6
KB4D-10	4.5 \pm 0.7	3.9 \pm 0.5	1.7 \pm 0.3

^a 5.0 \times 10⁴–1.0 \times 10⁵ cells/ml/well were seeded onto 24-well plates and allowed to grow for 48 h. The cells were washed with serum-free media then [³H]flutamide, [³H]hydroxyflutamide, or [³H]dihydrotestosterone were added and incubated for 30 min. Drug accumulation was measured as described in "Materials and Methods." Each value represents the mean \pm SD of quadruplicate determinations (^b, $P < 0.05$, ^c, $P < 0.01$ comparing the accumulation of drugs in MRP1-overexpressing cell lines to that of the parental controls).

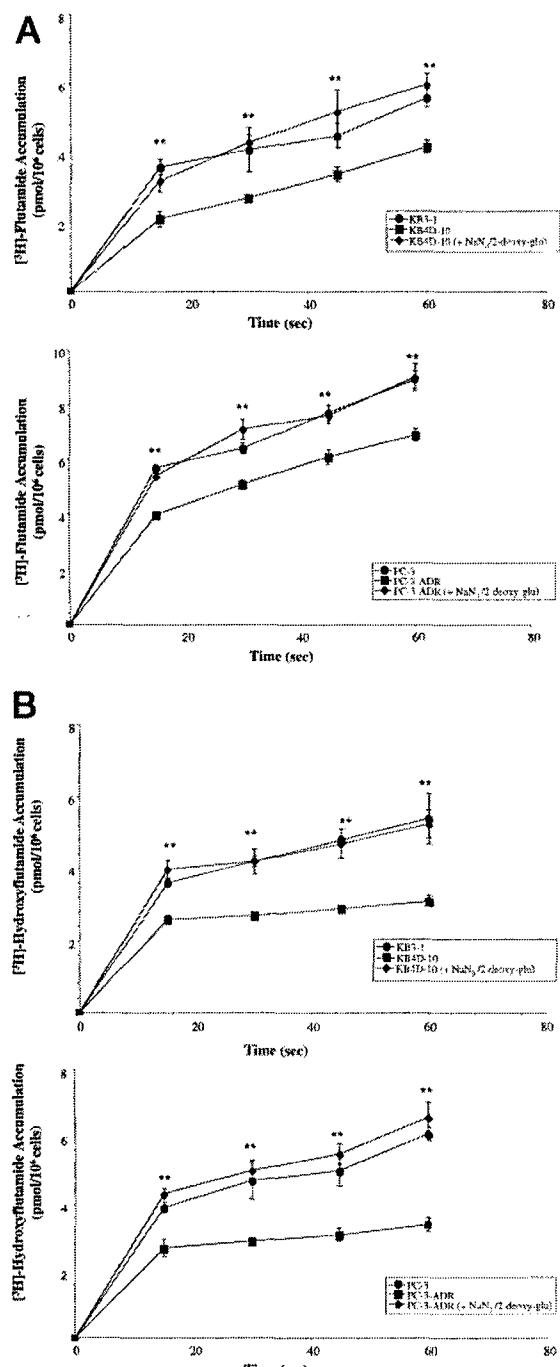


Fig. 4. Effect of sodium azide and 2-deoxy-D-glucose on flutamide and hydroxyflutamide accumulation. KB3-1 and KB4D-10 (top) and PC-3 and PC-3-ADR (bottom) were seeded onto 24-well plates. Forty-eight h later, the cells were incubated for 30 min in glucose-free media supplemented with 15 mM sodium azide and 50 mM 2-deoxy-D-glucose. The accumulation of (A) [3 H]flutamide or (B) [3 H]hydroxyflutamide was assayed as described in "Materials and Methods." Each point represents the mean \pm SD of quadruplicate determinations. The results are a representative of three separate experiments (*, $P < 0.05$; **, $P < 0.01$, MRP1 cells treated versus untreated).

Several lines of evidence indicate that MRP1 rather than other MRP family members or other ABC transporters mediate the transport of flutamide. First, P-glycoprotein was not detected in the cell lines (Fig. 2); second, flutamide transport was inhibited by leukotriene D4 (Fig. 7) and BSO (Fig. 8), inhibitors of MRP-family transporters (MRP1, MRP2, MRP3, and MRP6; Refs. 13, 17, 25); third, we excluded MRP2 and MRP3 as flutamide transporters because they are ex-

pressed in both the parental lines and resistant lines (26), yet BSO (Fig. 8), leukotriene D4 (Fig. 7), and VX-710 (Refs. 23, 24; Fig. 9) did not affect flutamide transport in parental cells. These data narrowed the possibility to MRP1 and MRP6. Western analysis revealed no differences in MRP6 expression in sensitive and resistant cell lines (data not shown). Finally, the KB4D-10 cells, which were transfected with a MRP1 expression vector, reproduced the effects on flutamide transport that was seen with the selected cell lines. Currently, there are no fully selective MRP1 substrates to use as a positive control.

Transport of both flutamide and its active metabolite hydroxyflutamide (27) are affected by MRP1 to a similar extent (Fig. 3). Therefore, hydroxylation by the cytochrome p450 enzyme CYP1B1 does

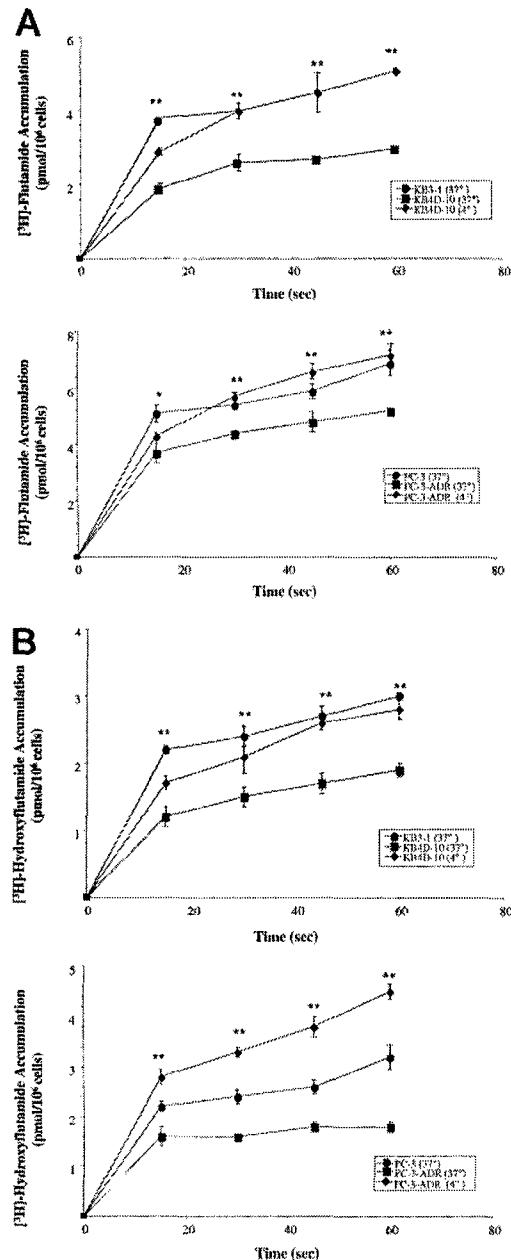
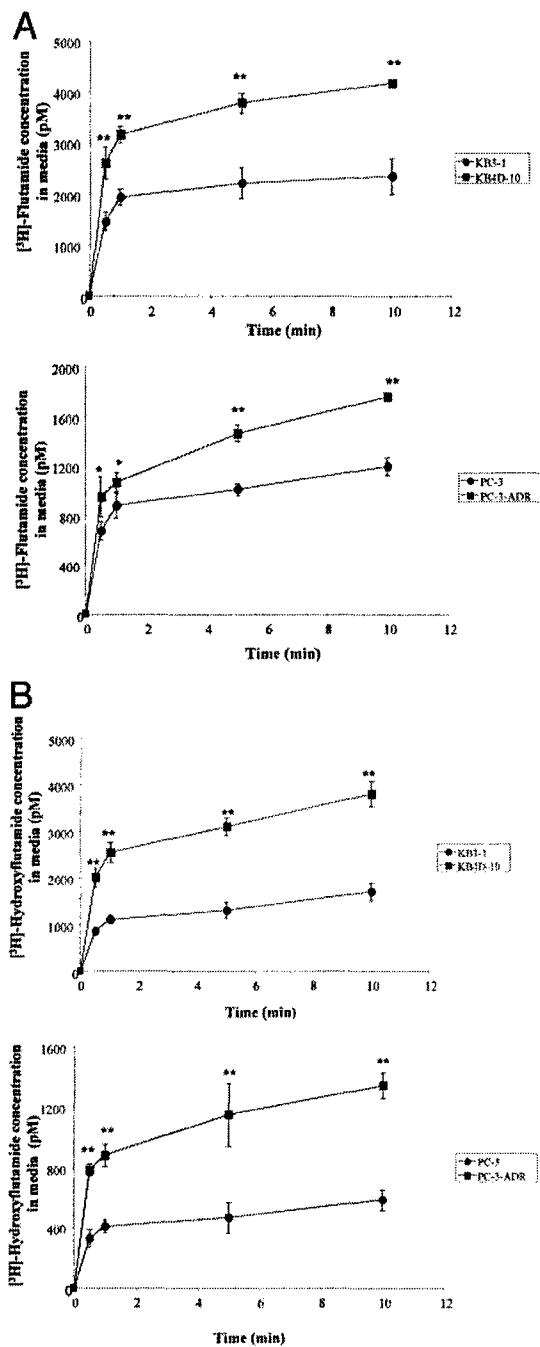


Fig. 5. Effect of temperature on flutamide and hydroxyflutamide accumulation. KB3-1 and KB4D-10 (top) and PC-3 and PC-3-ADR (bottom) were seeded onto 24-well plates. Forty-eight h later, the cells were incubated for 1 h at 4°C. The accumulation of (A) [3 H]flutamide or (B) [3 H]hydroxyflutamide was assayed as described in "Materials and Methods." Each point represents the mean \pm SD of quadruplicate determinations. The results are a representative of three separate experiments (*, $P < 0.05$; **, $P < 0.01$, MRP1 cells treated versus untreated).

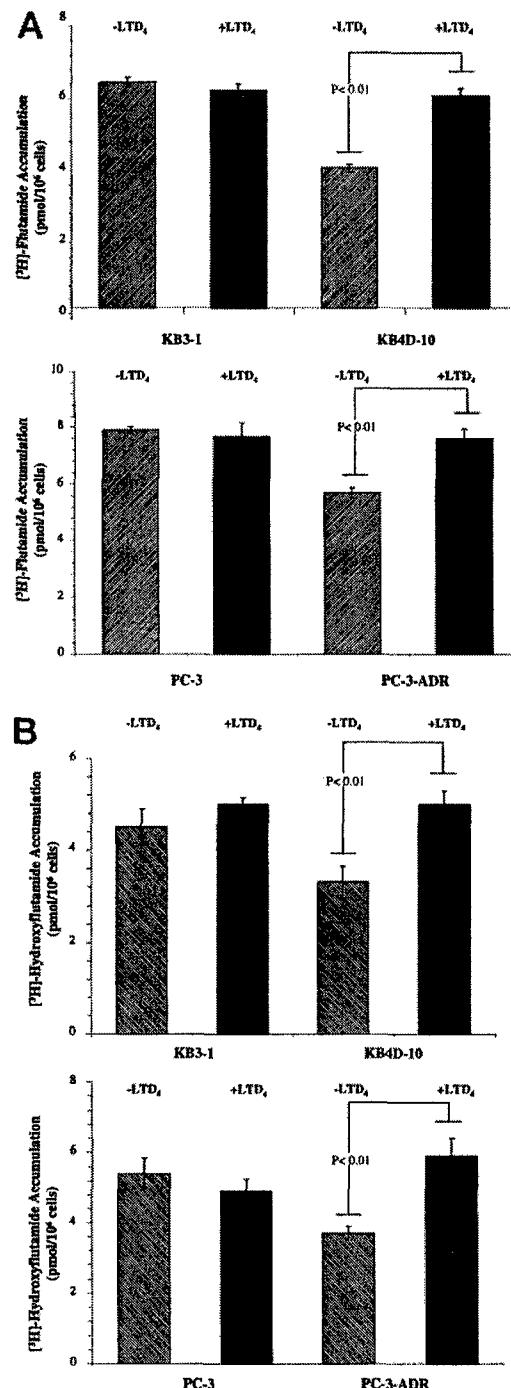


not influence transport of the active metabolite (28, 29). It was not possible to determine the effect of MRP1 overexpression on sensitivity of these cell lines to antiandrogen therapy because the cells are androgen independent, and insufficient quantities of hydroxyflutamide are available for cell viability studies.

Flutamide shares structural similarities with other MRP1 substrates. These characteristics include a negative charge on an aromatic ring containing a strong electron withdrawing group ($-CF_3$) (Fig. 1; Refs.

12, 13, 18). Other compounds that are transported by MRP1 that contain these structural features include the short-chain lipid 6-[N -(7-nitrobenz-2-oxa-1,3-diazol-4-yl) aminohexanoyl]-sphingosine glucosylceramide (30) and 2,4 dinitrophenyl-S-glutathione (16, 18).

The emergence of hormone-refractory prostate cancer is a major factor in the demise of patients with this disease. Our current studies build on previous results to create a scenario by which the function of



p53 may contribute to androgen independence. When studying the expression of drug resistance proteins in *de novo* human prostate cancer, we were struck by the coexpression of mutant p53 and MRP1 in surgical samples (10). We demonstrated that MRP1 expression is repressed by wild-type p53 and that expression is increased when p53 is inactivated by mutation (11). We found that dihydrotestosterone is not transported by MRP1 (Fig. 3C, Table 1), and these data suggest that in the presence of MRP1, dihydrotestosterone can still accumulate in cells and interact with androgen receptors, whereas the interaction

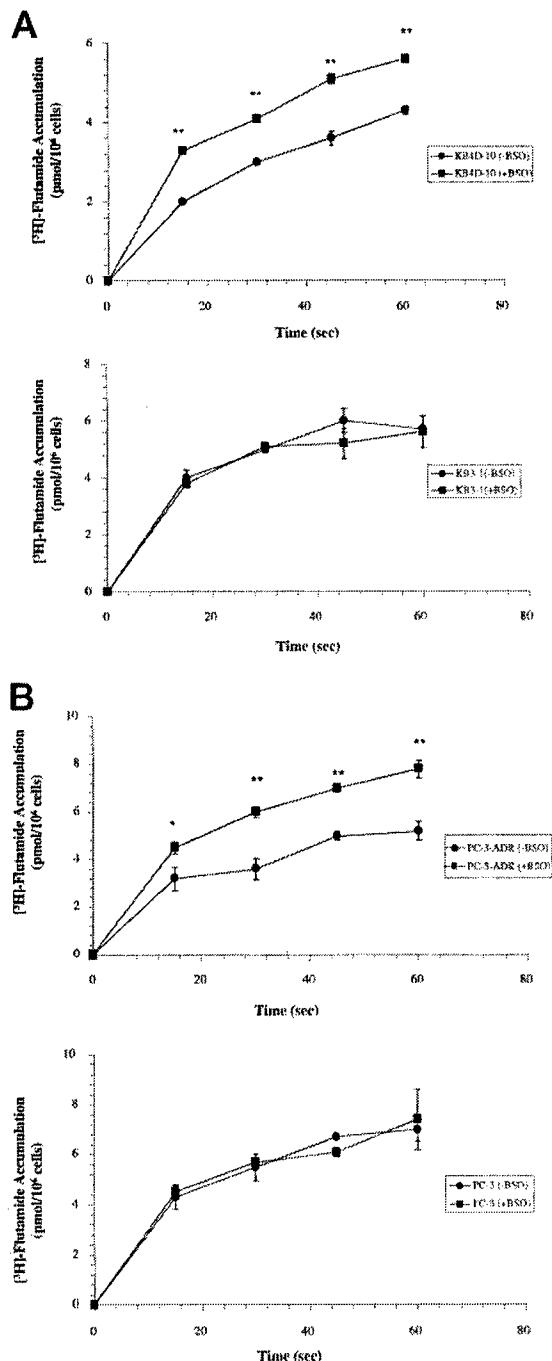


Fig. 8. Effect of glutathione depletion on the accumulation of flutamide. *A*, KB3-1 (bottom) and KB4D-10 (top) and *(B)* PC-3 (bottom) and PC-3-ADR (top) cells were seeded onto 24-well plates, and 50 μ M BSO added 24 h later. Drug accumulation was assayed as described in "Materials and Methods." Each point represents the mean \pm SD of quadruplicate determinations. The results are a representative of three separate experiments (*, $P < 0.05$; **, $P < 0.01$, MRP1 cells treated *versus* untreated).

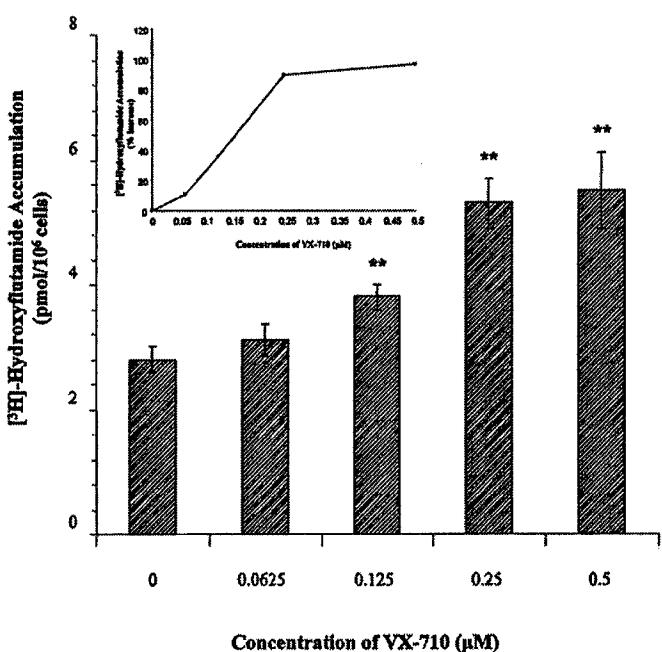


Fig. 9. Effect of VX-710 on the accumulation of hydroxyflutamide. PC-3-ADR cells were plated onto 24-well plates and allowed to grow for 48 h before VX-710 was added for 3 h. Drug accumulation was assayed as described in Fig. 4 and "Materials and Methods." The inset signifies the percentage increase over control (no addition of VX-710). Each point represents the mean \pm SD of quadruplicate determinations. The results are a representative of three separate experiments (*, $P < 0.05$; **, $P < 0.01$ MRP1 cells treated *versus* untreated).

of the antiandrogen with this target will be decreased. Our data suggest that in addition to the known effects of p53 on apoptosis (9), loss of p53 function may lead to resistance to antiandrogen drugs through MRP1-mediated drug transport.

In summary, we have shown that flutamide is transported by MRP1 based on drug accumulation and efflux studies and the ability of leukotriene D4, BSO, and VX-710 to restore steady-state concentrations. At the same time, cell lines that overexpress MRP1 retain baseline accumulation of dihydrotestosterone. These results suggest an unexpected role of MRP1 in the process of resistance to antiandrogen therapy of prostate cancer that could potentially be addressed through the use of MRP1 modulators.

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#1511 Overexpression of the Multidrug Resistance Protein (MRP) Produces Resistance to Estramustine and Flutamide. Matthew James Grzywacz, Jin-Min Yang, and William Hait. *The Cancer Institute of New Jersey: UMDNJ, Robert Wood Johnson Medical School, New Brunswick, NJ.*

We have recently shown that the expression of multi-drug resistance protein (MRP) in prostate and other cancers is regulated by the functional status of p53 (Sullivan et al., Jrnl. Clin Inv. 105: 1261-1267, 2000). However, little is known about the effect of MRP on drugs commonly used to treat prostate cancer. Therefore, we studied the effects of MRP on the sensitivity to flutamide and estramustine, two of the most commonly used agents for treatment. MDCKII canine kidney cells transfected with an MRP expression vector and its parental cell line were used. Cells (2×10^3 cells/100 μ l) were seeded in 96 well plates and incubated with increasing concentrations of estramustine (0-50 μ M) or flutamide (0-100 μ M). Cell viability was measured by MTT following a 48 hour incubation at 37°C. Cells that expressed MRP showed a 10-1000 fold increase in resistance to estramustine and flutamide as compared to the parental cells. These studies suggest that overexpression of MRP causes resistance to estramustine and flutamide. Since MRP is increased in a high percentage of prostate cancers, further studies will determine the clinical relevance of these observations.

#3685 The active metabolite of flutamide, 2-hydroxyflutamide, is a substrate for MRP1. Matthew Grzywacz, Jin-Ming Yang, and William Hait. *The Cancer Institute of NJ: UMDNJ-Robert Wood Johnson Medical School, New Brunswick, NJ.*

Prostate cancer is the most common non-cutaneous malignancy in American men. Although most cells require androgens for growth and development, eventually they become resistant to current androgen-deprivation therapy. We previously found that progression from benign epithelium to high-grade cancer involves upregulation of the multi-drug resistance protein, MRP1. In addition, we have shown that flutamide, a drug commonly used in androgen deprivation therapy, is a weak substrate of MRP1. Upon drug administration, flutamide undergoes extensive first-pass hepatic transformation into 2-hydroxyflutamide, which is thought to be the active component that targets the androgen receptor. The purpose of this study was to investigate whether the conversion of flutamide to hydroxyflutamide affects transport by MRP1. MRP1 over-expressing cell lines (KB4D-10 and PC-3-ADR), had markedly decreased accumulation and increased efflux ($p < 0.05$) of hydroxyflutamide as compared to the parental controls (Table 1). In contrast, dihydrotestosterone accumulation was not affected by MRP1, suggesting that binding of dihydrotestosterone to the androgen receptor remains intact in MRP1 positive cells and that the effect of hydroxyflutamide is diminished. ATP depletion with sodium azide and 2-deoxyglucose, and MRP modulators, VX-710 and leukotriene D4, inhibited hydroxyflutamide transport mediated by MRP1. The effects of MRP1 expression, ATP depletion, and modulators on cellular accumulation were similar to those seen with flutamide. These results suggest a possible mechanism of resistance of prostate cancer to androgen antagonists and may have clinical implications in prostate cancer treatment.

Table 1. Steady state accumulation of flutamide, hydroxyflutamide, and dihydrotestosterone

Cell Line	Flutamide	Hydroxyflutamide (pmol/10 ⁶ cells)	Dihydrotestosterone
PC-3	7.4 +/- 0.34*	4.6 +/- 0.6 *	1.1 +/- 0.1
PC-3-ADR	5.9 +/- 0.79	3.1 +/- 0.6	1.2 +/- 0.2
KB3-1	6.9 +/- 0.94 **	5.2 +/- 0.6	1.4 +/- 0.6
KB4D-10	4.5 +/- 0.74	3.9 +/- 0.5	1.7 +/- 0.3

5.0x10⁴-1.0x10⁵ cells/mL/well were seeded onto 24 well plates and allowed to grow for 48 hours. The cells were washed with serum-free media then [³H]-flutamide, [³H]-hydroxyflutamide, or [³H]-dihydrotestosterone were added and incubated for 30 minutes. Drug accumulation was measured as described in "Materials and Methods". Each value represents the mean +/- standard deviation

[Back](#)**Abstract Number: 2136**

Repression of multidrug resistance protein 1 (MRP1) by pharmacologic alteration in p53 function in human prostate cancer cells

Matthew J. Grzywacz, Jin-Ming Yang, William Hait. Cancer Institute of New Jersey, New Brunswick, NJ.

Multi-drug resistance protein 1 (MRP1) is a member of the family of ATP-binding cassette transporters and has been implicated in multidrug resistance (MDR). We previously found that wild-type p53 repressed expression of MRP1 in human prostate cancer cells, and p53 mutations increased expression of MRP1 (J. Clin. Invest. 105:1261-1267). MRP1 overexpression decreased accumulation of the anti-androgen flutamide but not dihydrotestosterone (Cancer. Res. 2003). Therefore, we determined the effect of compounds that alter p53 function on MRP1 expression. LVCaP cells were developed by transfection of LNCaP cells with a temperature-sensitive p53 mutant Val 138. At 38°C, p53 is functionally inactive and MRP1 is overexpressed. LVCaP cells at 38°C were exposed to CP-31398 (Pfizer) and PRIMA-1 (Biomol), two compounds reported to reactivate p53. MRP1 expression was analyzed by RT-PCR and Western blot. We also developed an ELISA assay to screen compounds that have similar structure to CP-31398 for p53 reactivation. Micro-titer plates were coated with wild-type p53 conformation antibody mAB1620 and blocked with 3% BSA. Mutant p53 expressing cell line 10(3)-R175H was treated with varying concentrations of drugs for 15 hours, then incubated overnight at 4°C. Wild-type p53 was detected with a rabbit polyclonal p53 antibody and the signal was developed using an HRP-conjugated antibody. P21 and mdm2 were detected using Western blot. We found that chlorpromazine, promazine, and trans-flupenthixol caused a 2-3-fold increase in wild-type p53 conformation. CP-31398 increased wild-type p53 conformation 6-10-fold in the same cell line, and promazine and chlorpromazine increased p21 in a dose-dependent manner. The expression of mdm2 was unaffected by these compounds. LVCaP cells exposed to CP-31398 at concentrations up to 40 µM for 12 hours showed a 5-fold increase in p21 and 10-fold decrease in MRP1. In contrast, PRIMA-1 has less effect on p21 induction and failed to decrease MRP1 expression. These data suggest that CP-31398 is a more effective inducer of p21 expression and MRP1 repression than PRIMA-1 in the LVCaP cell line and that compounds with similar structures and greater activity can be identified.

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